2009

STUDIES ON THE ROLE OF ACID SPHINGOMYELINASE AND CERAMIDE IN THE REGULATION OF TACE ACTIVITY AND TNFα SECRETION BY MACROPHAGES

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Krasimira Rozenova

Lexington, Kentucky

Director: Dr. Mariana N. Nikolova-Karakashian,

Associate Professor of Physiology

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

STUDIES ON THE ROLE OF ACID SPHINGOMYELINASE AND CERAMIDE ON THE REGULATION OF TACE ACTIVITY AND TNFα PRODUCTION BY MACROPHAGES

Acid Sphingomyelinase (ASMase) activity has been proposed to mediate LPS signaling in various cell types. This study shows that in macrophages, ASMase is a negative regulator of LPS-induced TNFα secretion. ASMase-deficient ($asm^{-/-}$) mice and isolated peritoneal macrophages produce several fold more TNFα than their wild-type ($asm^{+/+}$) counterparts when stimulated with LPS. The mechanism for these differences however is not transcriptional but post-translational.

The TNFα converting enzyme (TACE) catalyzes the maturation of the 26kD precursor (proTNFα) to the active 17kD form (sTNFα). In mouse peritoneal macrophages, the activity of TACE rather than the rate of TNFα mRNA synthesis was the rate-limiting factor regulating TNFα production. Substantial portion of the translated proTNFα was not processed to sTNFα; instead it was rapidly internalized and degraded in the lysosomes. TACE activity was 2 to 3 fold higher in $asm^{-/-}$ macrophages as compared to $asm^{+/+}$ macrophages and was suppressed when cells were treated with exogenous ceramide and SMase. In $asm^{-/-}$ but not in $asm^{+/+}$ macrophages, indirect immunofluorescence experiments revealed distinct TNFα-positive structures in close vicinity of the plasma membrane. $Asm^{-/-}$ cells also had higher number of EEA1-positive early endosomes. Co-localization experiments that involved inhibitors of TACE and/or lysosomal proteolysis suggest that in $asm^{-/-}$ cells a significant portion of proTNFα is sequestered within the early endosomes, and instead of undergoing lysosomal proteolysis it is recycled to the plasma membrane and processed to sTNFα.

KEYWORDS: Acid Sphingomyelinase, Ceramide, TACE, TNFα, macrophages

Student’s signature

Date

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By

Krasimira Rozenova

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Director of Dissertation

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DISSERTATION

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CHAPTER ONE

Introduction

A. Regulation and biological effects of Tumor Necrosis Factor α in health and disease

Tumor necrosis factor α (TNFα)[1] belongs to a class of proteins, called cytokines, which are important mediators in host response to stresses such as infection, inflammation, and injury. It is highly conserved among mammals with 78% identity between human and mouse protein.

Since it was identified more than 30 years ago, several thousand papers looking mostly at biological effects of TNFα in variety of cell types and animal models have been published. TNFα was initially described as a factor capable of killing tumor cells *in vitro* and of causing hemorrhagic necrosis of transplantable tumors in mice [1]. Later studies revealed that it has a broad spectrum of biological activities including stimulation of neutrophil adhesion and activation, chemotaxis, phagocytosis, and vascular permeability to name of few [2-5].

[1] Abbreviations: ADAM, A Disintegrin and metalloproteinase; ANOVA, Analysis of Variance; ASMase, Acid sphingomyelinase; AP-1, Activator protein-1; EEA1, Early endosomal antigen 1; ELISA, Enzyme-linked immunosorbent assay; ERK, Extracellular signal regulated kinase; FITC, Fluorescin isothiocyanate; HPLC, High pressure liquid chromatography; IL-1β, Interleukin-1β; i.p., intraperitoneally; IRAK, Interleukin-1 receptor-associated kinase; JNK, c-Jun terminal kinase; LBP, LPS binding protein, LPS, Lypopolysaccharide; MAPK, Mitogen-activated protein kinase; MyD88, Myeloid differentiation factor 88; NBD, 6-((N-(7nitrobenz-2-oxa-1,3-diazol-4-yl))amino)-C_6; NF-κB, Nuclear factor κB; NH_4Cl, Ammonium chloride; NSMase, Neutral sphingomyelinase; PGE_2, Prostaglandin E2; PMA, Phorbol 12 myristate 13 acetate; RA, Rheumatoid arthritis; RFU, Relative fluorescent units; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA, Small inhibitory RNA; SM, sphingomyelin; SPT, serine palmitoyltransferase; S-1-P, sphingosine-1-phosphate; TACE, TNFα converting enzyme; TAK1, Transforming growth factor-β-activated kinase; TGFα, Transforming growth factor α; TLR, Toll-like receptor; TNFα, Tumor necrosis factor α; TRAF6, Tumor necrosis factor-associated factor; TRITC, Tetramethylrhodamine isothiocyanate.

Tissue and cellular effects of TNFα

TNFα has a central role in the initial host response to infection and it is a key player in the regulation of immune homeostasis. Studies in TNFα transgenic and TNFα *knockout* mice have demonstrated that TNFα has important immunomodulatory functions both *in vivo* and *in vitro*. It is required not only for the initial priming phase of antigen specific immunity and effector functions, but also for the resolution phase [6]. Furthermore, studies in TNFα *knockout* mice show that TNFα plays a critical role in germinal centers formation in the spleen [7]. These structures are important for antibodies isotype switching, affinity maturation and memory formation – thus linking TNFα to the adaptive immunity. TNFα also has diverse effects upon the neutrophil population ranging from stimulation of neutrophil release from bone marrow to regulation of neutrophil chemotaxis [4], degranulation [2], superoxide production [8], and lysozyme release [2]. TNFα stimulates the phagocytic potential of macrophages and the production of a number of pro- and anti- inflammatory mediators such as IL-1β, IL-6, PGE_2, etc [9] [10].

TNFα can induce a wide variety of biological effects and has a profound impact on the function of many cell types and tissues, which do not belong to the immune system. The incubation of endothelial cells with TNFα increases vascular permeability [11]. Chronic delivery of TNFα has been linked to the loss of total body protein and significant histologic changes within the liver [12]. Furthermore, *in vivo* studies indicated that sub-lethal doses of TNFα affect the adipose tissue and elevate serum levels of triglycerides and free fatty acids [13, 14].

Dysregulation of TNFα and Diseases

In a healthy state, TNFα is produced in response to various stimuli mainly by macrophages. However, the onset of many diseases is correlated with the

induction of TNFα production by various cells that normally do not produce it. Interestingly, however, the important questions of how it is regulated and what makes the difference between its beneficial and deleterious effect are not yet answered. Nevertheless, there are three major factors which appear to be of significant importance: concentration, duration and location, or combinations of them.

TNFα is not usually detectable in young and healthy individuals, but its levels sharply increase during inflammatory response. Importantly, its secretion is transient and TNFα is quickly and efficiently cleared when the pathogen is neutralized. The importance of the strict regulatory mechanisms sensing and maintaining proper TNFα levels is underlined by the observation that pathological dysregulation of TNFα production/clearance occurs in many diseases.

Uncontrolled systemic release of TNFα is a hallmark of septic shock and it is tightly linked to the morbidity and mortality of the disease [15, 16]. Unlike systemic inflammation, TNFα in rheumatoid arthritis (RA) is produced locally, but its secretion persists over a long period of time. Similarly, local but chronic production of TNFα is implicated in the pathology of Crohn's disease and asthma. The cause-effect relationship between TNFα and the onset or the severity of the disease is supported by experimental data showing that overexpression of TNFα in mice results in chronic inflammatory arthritis and inflammatory bowel disease [17, 18].

TNFα is mostly discussed in the context of the innate immunity but it can also be produced by cells unrelated to the immune system, such as adipocytes, smooth and cardiac muscle cells, endothelial cells, osteoclasts, etc (Fig.1.1). Mounting evidence suggests that obesity promotes a state of chronic inflammation with elevation of TNFα, which contributes to insulin resistance and type 2 diabetes. Although it is not entirely clear what the trigger of TNFα production is for the prediabetic state, it might be nutritionally regulated.

Dysregulation of TNFα production and chronic inflammation are also a hallmark of physiologic process such as aging [19]. TNFα is one of the prime signals inducing cellular apoptosis in muscle, and is therefore an important

contributor to muscle waste in the elderly [20, 21]. Elevated levels of TNFα are also evident in a large number of neurological disorders including: traumatic brain injury, multiple sclerosis, Alzheimer's disease, and Parkinson's disease; but whether TNFα signaling actively contributes to, or limits neuronal injury in these disorders has yet to be established [22-25].

On certain occasions chronic elevation of TNFα may prove beneficial in suppressing the active stage of a disease. For instance, the effects of TNFα blockade, which is beneficial in a number of diseases has been assessed in individuals with multiple sclerosis and was found to worsen the disease [26, 27]. Furthermore, immunosuppressive effects of TNFα might explain the reports of rare demyelinating syndromes in patients after prolonged TNFα blockade [28], as well as the development of antinuclear antibodies and lupus syndromes in a significant proportion of patients with RA receiving anti-TNFα therapy [29].

At the cellular level, TNFα activates NF-κB, JNK, ERK, and caspases, which are part of signaling pathways leading to cell survival, proliferation, and apoptosis. It has been suggested that acute and chronic TNFα responses may be determined at the biochemical level by the complex interactions between these pathways [30]. For example, TNFα plays an important role in immune homeostasis including lymphocyte activation, survival, and differentiation. Inhibition of the caspase pathway by sustained activation of NF-κB will generate a survival signal, which is critical for rapid immune responses to foreign pathogens. On the other hand, inappropriate expansion of self-reactive lymphocyte population at sites of inflammation would clearly be detrimental. The factors which act as a switch between pro-survival and pro-apoptotic pathways still remain mostly unknown. However, a study in human fibroblasts points at NF-κB as such a factor, demonstrating that prolonged stimulation with TNFα impairs IκBα re-synthesis, resulting in continued activation of NF-κB response [31].

B. Sphingolipids metabolism and biological effects

Sphingolipids are essential components of all biological membranes, but are also well-recognized active biomolecules which mediate and modulate diverse signaling pathways. Sphingosine is one of the first sphingolipids which was identified, and has been implicated in the regulation of cell cycle, cytoskeleton reorganization, endocytosis, and apoptosis [32]. Even more attention has been given to two other sphingolipids, ceramide and sphingosine-1-phosphate (S-1-P), which often have antagonistic effects: ceramide regulates many cell-stress responses including apoptosis and cell senescence [33, 34], while S-1-P has a crucial role in cell survival and cell migration [35]. Among these bioactive sphinglipids, ceramide is produced in the largest amount and is considered the central molecule in sphingolipid biosynthesis and catabolism. The two main metabolic pathways for generation of ceramide are (i) the *de novo* synthesis in the endoplasmic reticulum, and (ii) the turnover of sphingomyelin (SM) either at the plasma membrane or in the endosomal/lysosomal compartment (Fig. 1.2). A number of agonists have been shown to activate these pathways leading to transient elevation in ceramide. The magnitude and temporal pattern of ceramide accumulation is further influenced by the activity of ceramidases, SM synthases, ceramide kinase, and glucosyl/galactosyl ceramide synthases. These enzymes catalyze the conversion of ceramide to other sphingolipids, and some agonists seem to coordinately regulate both the ceramide production and turnover.

The *de novo* pathway for synthesis of ceramide takes place in the endoplasmic reticulum and starts with condensation of palmitoyl-CoA and serine. This is the rate-limiting step catalyzed by serine palmitoyltransferase (SPT), which produces 3-ketosphinganine. Additional reactions of reduction followed by acylation generate relatively inactive dihydroceramide, which is converted by desaturase to the bioactive ceramide. The newly generated ceramide is actively transported to the Golgi apparatus, where it serves as a rate-limiting substrate in the synthesis of complex sphingolipids, like SM and glycosphingolipids.

The alternative pathway for ceramide generation is the hydrolysis of sphingomyelin by a group of enzymes known as sphingomyelinases (SMases). SMase activities with neutral and acidic pH optima are found in most mammalian

cells, and an enzyme active in alkaline pH is localized in the intestinal wall. Currently, research is focused on 4 genes encoding different mammalian SMases: *smpd1* encodes two forms of acidic SMase, one associated with the endosomal/lysosomal compartment (ASMase), and a second one found in the plasma and the conditioned medium of stimulated cells (SSMase). *smpd2* and *smpd3* encode the Neutral SMase 1 (nSMase1) and 2 (nSMase2), both of which are Mg^{2+}-dependent but differ in their sub-cellular localization and role in signaling. The recently cloned *smpd4*, is suggested to encode a novel form of NSMase, nSMase3 that is found predominantly in skeletal muscle and heart.

ASMase is a 72kD glycoprotein with a 57kD peptide core. The processing of the carbohydrate moieties within the Golgi is critical for sub-cellular trafficking of this enzyme. For example, the addition of mannose-6-phosphate residues is essential for lysosomal localization of ASMase. Alternative carbohydrate processing within the Golgi can result in targeting the enzyme to the secretory pathway [36, 37].

ASMase was first purified from human brain, and was found to have optimal pH of 5.0 [38]. Based on trypsin digestion and mass spectrometric analysis a model was proposed, according to which the enzyme has three domains: an activator domain, followed by a proline rich domain, and a catalytic C-terminal domain. Both the lysosomal and the secretory forms require divalent cation Zn^{2+} for proper activity [36]. Many lipids were also found to influence ASMase activity. For example, the presence of fatty acids, mono-, di-, and tri-acylglycerols as well as phosphatidylinositol has been found to activate ASMase and stimulate sphingomyelin hydrolysis [39]. On the other hand, ceramide-1-phosphate, S-1-P, and phosphatidylinositol-4,5-bisphosphate inhibit ASMase [40, 41]. Interestingly, cholesterol, which physically associates with sphingomyelin within lipid rafts, also inhibits ASMase [42]. Furthermore, the activation of ASMase leads not only to sphingomyelin hydrolysis but also cholesterol depletion [43]. Accordingly the loss of ASMase activity causes accumulation of both sphingomyelin and cholesterol.

ASMase is activated by numerous stimuli including TNFα, LPS, Fas/CD95, radiation, and ischemia, and in a large variety of cell types [44-49] (Fig.1.3). In many cases, the activation of ASMase is associated with its translocation to the plasma membrane [50]. The cellular mechanisms regulating ASMase activity and localization remain unclear, although it has been suggested that phosphorylation of Ser^{508} is required for ASMase translocation to the plasma membrane after UV irradiation of MCF-7 cells [51]. Furthermore, Jin et al demonstrated that lysosomal trafficking and fusion with the plasma membrane is linked to the formation of lipid rafts in endothelial cells [50]. Lipid rafts have been implicated in receptor clustering and initiation of signal transduction via death receptors such as TNFR and CD95 [44, 46, 52]. Data coming from the inhibition of ASMase activity (using siRNA, or pharmacologic inhibitors or using NPD cells or *knockout* mice) have shown that this enzyme is indispensable for the execution of apoptotic response in variety of cell types. For instance, ASMase *knockout* mice were protected against endothelial apoptosis and had higher survival rate in response to LPS [53]. Furthermore, studies in fibroblasts from Niemann–Pick disease type A patients, who exhibit deficiency of ASMase and hepatocytes from ASMase *knockout* mice show that ASMase activation is required and sufficient for CD95 induced apoptosis [46]. ASMase is also important for tumor suppression and sensitivity to radiation therapy. Tumors growth progresses faster in ASMase *knockout* mice compared to the wild-type controls.

The hydrophobic nature of ceramide limits its diffusion in the water-based cellular environment, and therefore its action is limited to the location of its generation, mainly endolysosomes and lipid rafts. Only a few direct targets of ceramide have been described, which can potentially serve as mediators of its action. Lysosomal protease cathepsin D is one of them. After its activation by ASMase derived ceramide, the mature form of cathepsin D is released in the cytosol and triggers mitochondrial death pathway [54].

ASMase has an important role in pathogen infection and recovery. ASMase *knockout* mice easily succumb to infections with *P. aeruginosa*, *L*

monocytogenes, and alphaviruses [55, 56]. However, during bacterial or viral infections the role of ASMase is most likely linked to membrane reorganization and impact on internalization and vesicle fusion rather than modulation of signaling pathways. ASMase deficient cells are incapable of *P.aeruginosa* internalization and apoptosis, which are essential to clear the pathogen. The mechanism underlying high susceptibility of ASMase *knockout* mice to *L. Monocytogenes* revealed that a lack of ASMase activity leads to impaired phago-lysosomal fusion, which is required for lysosomal degradation of the bacteria [57]. The role of ASMase activity in viral infections is very diverse. It appears to be important for the uptake of rhinoviruses as well as the infection and replication of herpes virus [58]. Ng et al demonstrated that after infection with Sindbis virus ASMase deficient cells produced more infectious virions compared to the control cells [59].

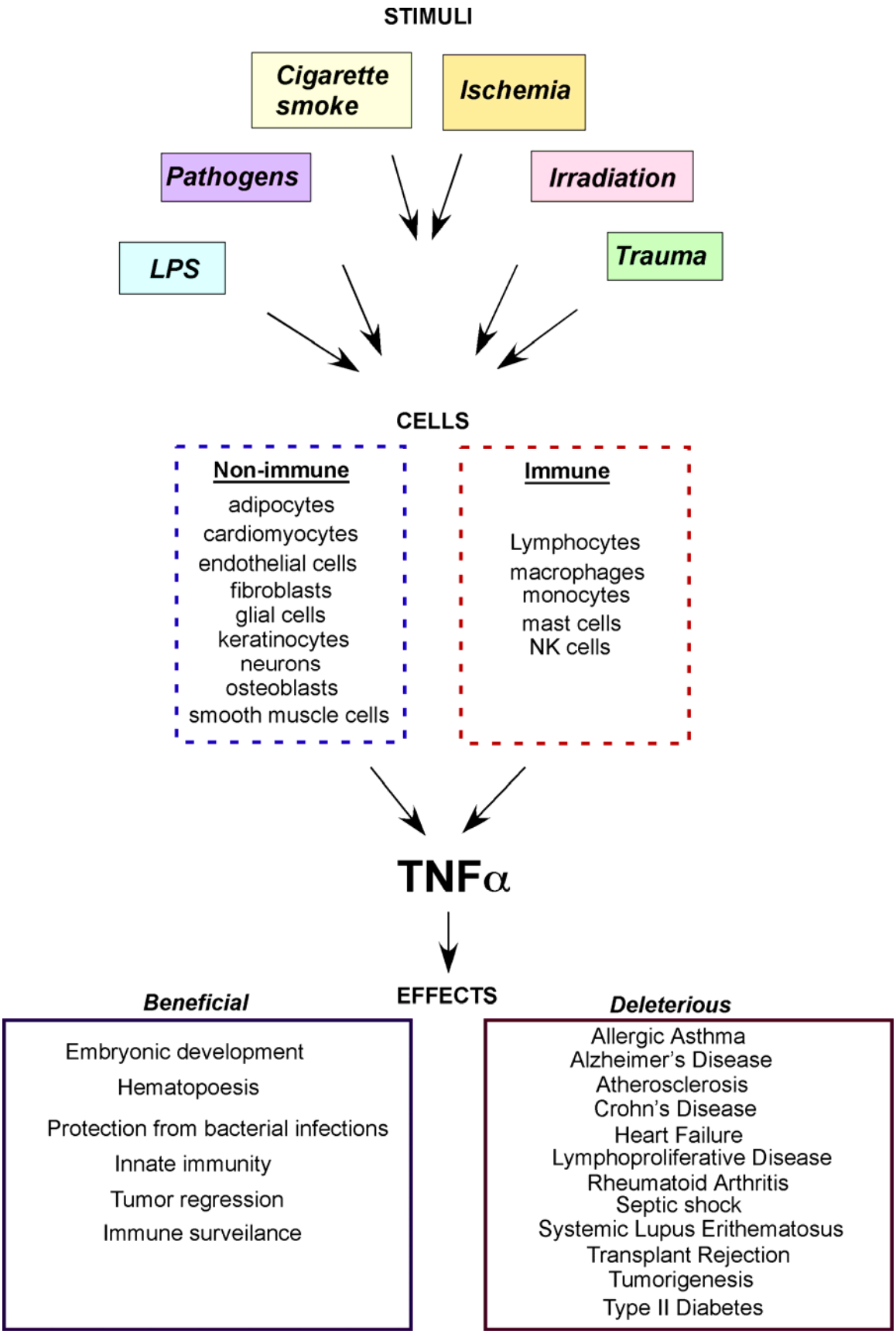

STIMULI
Cigarette smoke
Ischemia
Pathogens
Irradiation
LPS
Trauma
CELLS
Non-immune
adipocytes
cardiomyocytes
endothelial cells
fibroblasts
glial cells
keratinocytes
neurons
osteoblasts
smooth muscle cells
Immune
Lymphocytes
macrophages
monocytes
mast cells
NK cells
TNFα
EFFECTS
Beneficial
Embryonic development
Hematopoesis
Protection from bacterial infections
Innate immunity
Tumor regression
Immune surveilance
Deleterious
Allergic Asthma
Alzheimer's Disease
Atherosclerosis
Crohn's Disease
Heart Failure
Lymphoproliferative Disease
Rheumatoid Arthritis
Septic shock
Systemic Lupus Erithematosus
Transplant Rejection
Tumorigenesis
Type II Diabetes

Figure 1.1. Summary of the stimuli activating TNFα producing cells and subsequent biological effects initiated by TNFα.

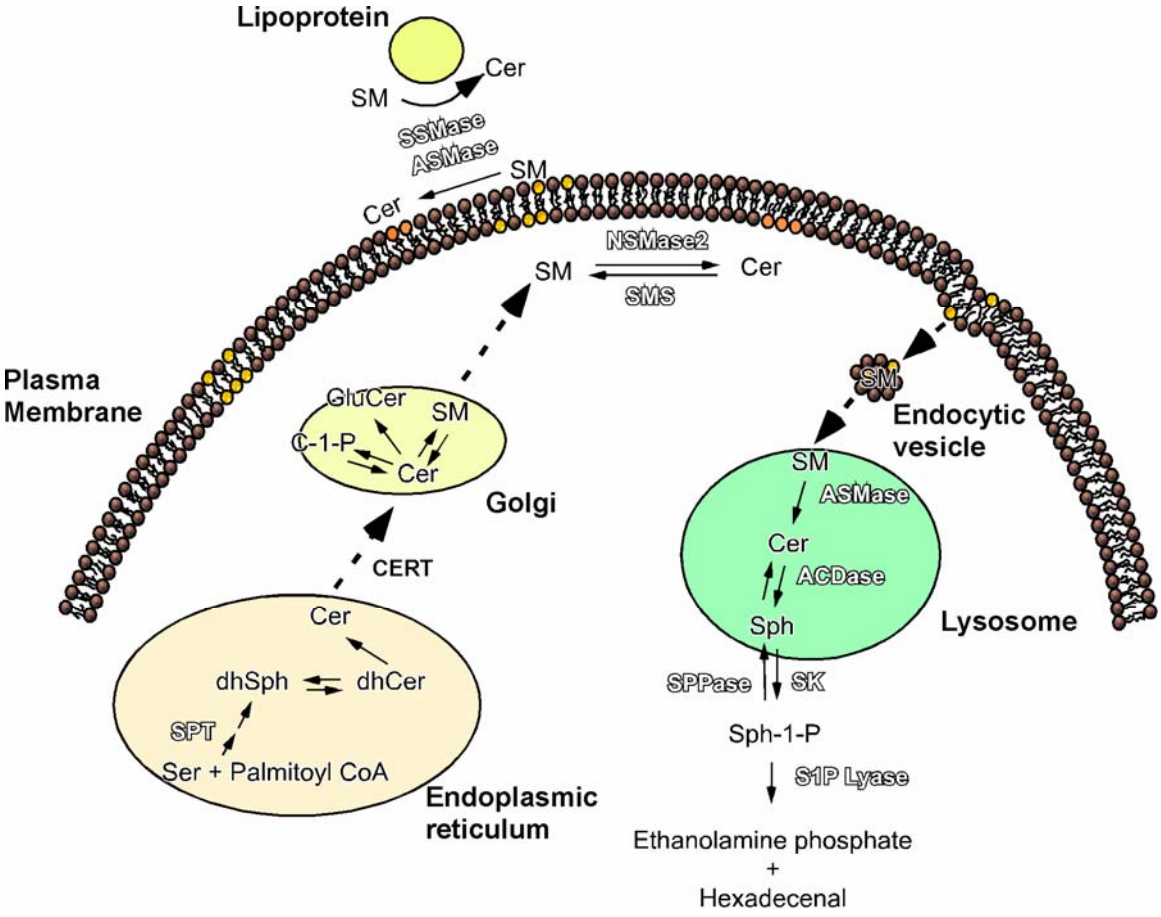


Figure 1.2. Metabolic pathways responsible for ceramide synthesis and degradation. The names of relevant subcellular organelles are shown in bold. Black solid arrows are used to depict metabolic conversions. Black dashed arrows indicate protein-mediated or vesicular transfer. *Abbreviations*: ACDase, Acid Ceramidase; ASMase, Acid Spjingomyelinase; Cer, ceramide; C-1-P, ceramide-1-phosphate; dhCer, dihydroceramide; dhSph, dihydrosphingosine; NSMase2, neutral sphingomyelinase 2; SK, sphingosine kinase; SM, sphingomyelin; Sph, sphingosine; SMS, sphingomyelin synthase; SPPase, sphingosine phosphate phosphatase; SPT, serine palmitoyltransferase; SSMase, secretory sphingomyelinase

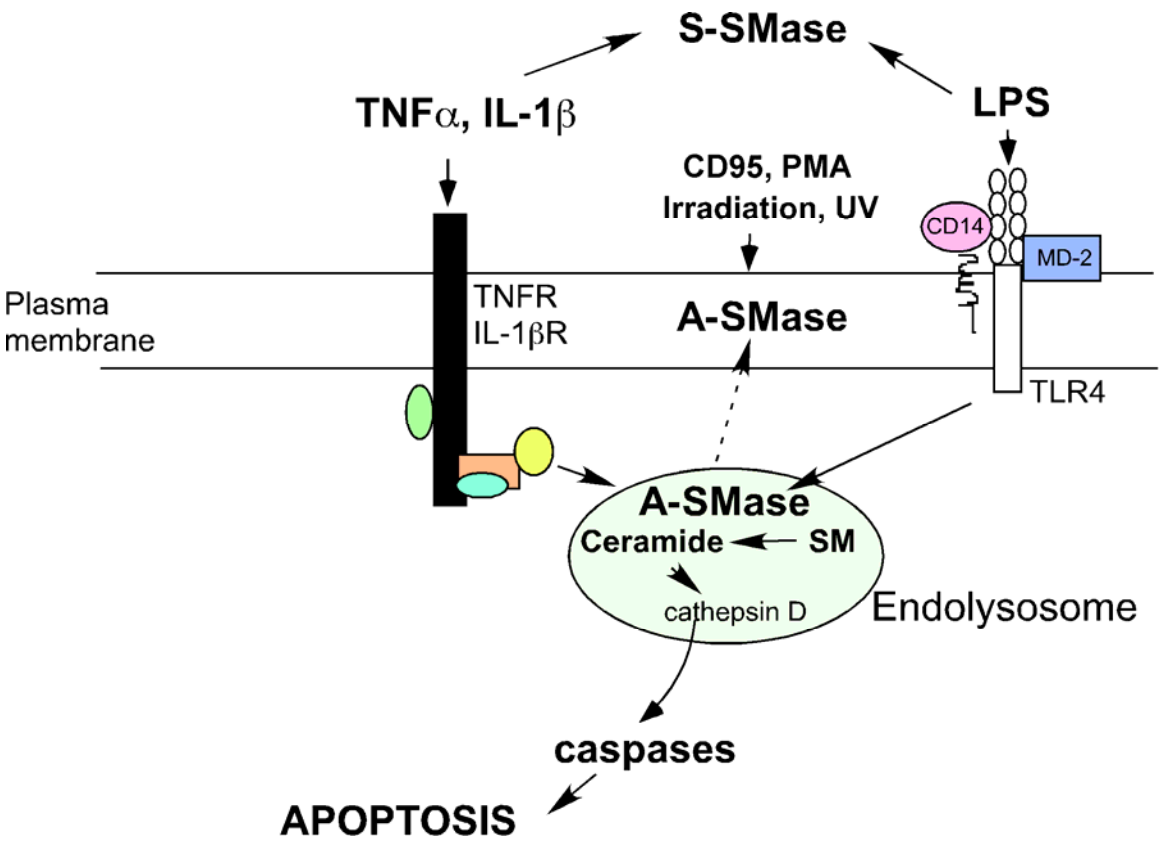


Figure 1.3. Agonists and signaling pathways for activation of Acid and Secretory sphingomyelinases.

CHAPTER TWO

LPS-induced production of TNFα by macrophages. *In vivo* and *in vitro* models to study the role of ASMase in LPS signaling

A. Introduction

Macrophages play a pivotal role in the production and regulation of TNFα levels. TNFα is one of the first pro-inflammatory cytokines secreted in response to bacterial infection and appears to mediate the majority of the symptoms seen in sepsis [60, 61]. The rate of TNFα biosynthesis increases 1000 fold or more following stimulation with LPS, which makes it one of the most potent inducers of TNFα production by macrophages [62].

LPS is a component of the outer membrane of gram-negative bacteria and is indispensable for the integrity of the bacterial membrane. It has been estimated that one bacterial cell contains approximately $3.5x10^6$ LPS molecules, which occupy three-quarters of the bacterial surface [63]. LPS consists of 3 components: lipid A, core oligosaccharide, and polysaccharide side chain (O-specific chain). Lipid A is the active component recognized by the innate immune system [64].

In the circulation, LPS is present in a complex with LPS binding protein (LBP). The recognition of LPS at the cell surface is required for the initiation of the transmembrane signal leading to cell activation, and involves at least 3 proteins: CD14, MD-2, and TLR4. CD14 can exist as a soluble molecule, but is more often anchored to the plasma membrane by a glycosylphosphatidylinositol anchor. It facilitates the transfer of LPS from LBP to a TLR4/MD-2 receptor complex [65, 66]. MD-2 is an indispensable molecule in the LPS sensing complex and physically associates with the signaling receptor TLR4. Dimerization of the receptor initiates the signal transduction by the engagement of cytosolic adaptor proteins. MyD88-dependent signaling cascade is well-studied and depends on the phosphorylation and proteosome-mediated

degradation of IRAK-1. This is crucial for the activation of downstream targets such as MAP kinases and transcription factors including NF-κB, AP-1, Ets, and Elk-1 [67]. *In vitro* stimulation of macrophages with LPS results in a number of changes in the transcriptional and translational machinery of the cell, which favor the production and release of TNFα. Transcription of the TNFα gene leads to the production of a single mRNA product. The control of TNFα-gene expression is mediated primarily by NF-κB binding sites present within the TNFα gene promoter [68-71], although the requirement of raf-1/MEK-1/2/ERK1/2 has been also suggested by number of studies [62, 72]. It has been found that treatment of cells with ERK inhibitor PD-098059 reduced TNFα mRNA expression in a dose dependent manner and TNFα secretion was completely abolished in the presence of Ro 09-2210, a MEK inhibitor, which blocks not only activation of ERK1/2, but also JNK and p38 [73]. Additional studies further supported the role of p38 activation for TNFα production [74] and also suggested that JNK might be involved in the translational regulation of TNFα [75]. Translational activation of TNFα is dependent on a conserved element found within the 3' untranslated regions [76]. Translation of mouse TNFα mRNA produces the 26kD TNFα precursor, which has 233 amino acids and undergoes post-translational glycosylation giving rise to several modified species which are susceptible to treatment with glycosylase inhibitors and contribute to the smear like pattern seen on a Western blot [77]. An additional form of sTNFα with an estimated molecular weight of 19kDa has been observed and was shown to contain additional amino acids from the precursor protein as a result of different post-translational processing [78]. Glycosylation of TNFα has not been detected in human samples and its physiological importance in mouse is currently unknown. However, it was determined that it does not interfere with the biological activity of this cytokine *in vitro* [78].

The TNFα precursor contains a highly conserved sequence of 79 amino acids at the N-terminus, which serves to anchor proTNFα in the plasma membrane. The precursor is a type II membrane protein with 18 amino acids comprising the hydrophobic transmembrane region. The 26kD form is

proteolitically cleaved by the TNFα converting enzyme (TACE) to give a 17kD active form. sTNFα exists in solution as a homotrimer with a total molecular weight of 52KD. This is the form that binds to the TNFα receptors and exerts its biological effects.

LPS also activates the secretory and lysosomal forms of Acid sphingomyalinase, one of the major ceramide producing enzymes. Importantly, accumulation of ceramide after LPS stimulation has been also reported and coincides with the peak of ASMase activity. ASMase is proposed to mediate many of the cellular effects of LPS, including the induction of apoptosis in endothelial cells, hepatic necrosis, etc [53]. Although the effects of ceramide on LPS-induced signaling molecules are various and often depend on the cell type, ASMase derived ceramide appears to play an important role as a modulator of LPS response. Whether ASMase affects TNFα production is unclear, however such effect is likely because ASMase is proposed to mediate the activation of NF-κB and JNK in response to other agonists.

In humans, a genetic deficiency of ASMase results in autosomal recessive Niemann-Pick disease (NPD), which is further divided into type A and B based on clinical and pathological findings. Mutations in the ASMase gene cause an abnormal accumulation of sphingomyelin and in patients with the more severe form of the disease (type A) this affects the function of visceral organs and brain, and leads to lethality at early age. Fibroblasts from patients with NPDA are often used to study the role of ASMase in various cellular responses.

A valuable tool to study the enzyme functions *in vivo* is the colony of ASMase *knockout* mice, which lack both the lysosomal and secretory forms of ASMase These mice also serve as source for a variety of cell types lacking ASMase activity and represent an animal model for the human disease since at age of 4 months they develop the neurodegenerative phenotype seen in NPDA patients.

Acute inhibition of ASMase has been achieved by the tricyclic amines such as desipramine and amitriptyline and is also frequently used in cell culture models. These amphiphilic drugs displace the enzyme from lysosomal membranes, making it vulnerable to proteolytic degradation [79].

The major goal of the studies presented in this chapter is to develop an experimental system *in vivo* and *in vitro* to study the regulation of TNFα production in response to LPS, as well as to decipher the role of ASMase and ceramide in these regulatory mechanisms.

B. Materials and Methods

Materials

LPS (*E. coli,* Serotype 026:B6), Desipramine hydrochloride, and Brewer thioglycollate broth were purchased from Sigma-Aldrich Co. (St. Luis, MO). The enhanced chemifluorescent kit was from GE Healthcare (Piscataway, NJ). Antibodies were from the following manufacturers: anti-phospho-ERK1/2, and anti-IRAK-1 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-TNFα anti-cyclophilin A were from Cell signaling (Beverly, MA) anti-β-actin and alkaline phosphatase conjugated secondary antibodies were from Sigma-Aldrich Co. (St. Luis, MO). Trizol® and Superscript II™ reverse transcriptase were from Invitrogen (Carlsbad, CA). Random hexamers were from Roche (Indianapolis, IN). *Taq* DNA polymerase was from New England Biolabs (Ipswich, MA). RNeasy Mini Kit and siRNAs (scrambled and ASMase-specific) were from Qiagen (Valencia, CA).

Animals

C57BL/6 mice were maintained in the AAALAC-approved animal facility of the University of Kentucky Medical Center and placed on a standard NIH-31 diet, with a 12-h light/dark cycle in microisolation. Mice were injected *i.p.* with LPS (5.8mg/kg b.w.) or an equivalent volume of 150mM NaCl. Blood was collected by cardiac puncture and serum was obtained in serum separator tubes.

Cell cultures and treatment

RAW264.7 cells were maintained in DMEM supplemented with 100 units/ml penicillin/streptomycin (Gibco Laboratories, Grand Island, NY) and 10% heat inactivated FBS. Cells were plated in 35mm dishes and used for experiments at a density of approximately $2x10^6$ cells/dish.

Two different ASMase-specific siRNAs (Quigen#SI00200753 and [80]) were used for silencing of the endogenous ASMase expression. BLAST search confirmed that the sequences were specific for the *smpd1* gene. Cells were plated in DMEM at density $0.7x10^6$ cells/dish and transfections (160 pmol of siRNA per dish) were performed 16 hours later using Lipofectamine™ according to the manufacturer's instructions. Control plates were transfected with non-silencing scrambled siRNA. Transfection efficiency was monitored after transfection with Alexa Fluor555-labeled scrambled siRNA.

Treatments with LPS were done at 19 hours after plating. Where indicated, cells were pretreated for 2 hours with 25μM Desipramine.

Peritoneal macrophages were elicited with thioglycollate from 8 weeks old $asm^{+/+}$ mice. Cells were plated in DMEM, and supplemented with 2% heat inactivated FBS on 6-well plates at a density of $2x10^6$cells/well. Macrophages were allowed to adhere for 3 hours in a 37°C humidified 5% CO_2 incubator and non-adherent cells were removed by aspiration.

Human fibroblasts from healthy individuals and patients with Niemann-Pick Disease Type A were maintained in MEM supplemented with 15% FBS. Cells were plated in 60mm dishes and used for experiments at density of approximately 10^6 cells/dish. Cells and medium were used for Western blotting 48 hours following transfection.

SDS-PAGE and Western blotting of medium and cell lysates

Conditioned medium was collected from wells, each containing $2x10^6$ cells, cleared by centrifugation, and concentrated ten fold using Amicon Ultra tubes with a cutoff of 10kD. Ten microliters from the concentrated medium were subsequently used for Western blotting. Cells were lysed on ice for 30 min in a buffer containing 1mM EDTA, 1% Triton X-100, 1mM Na_2VO_4, 1mM NaF, and a protease inhibitor cocktail (1:200) in 10mM Tris-HCl, pH 7.4. Cell debris was removed by centrifugation at 16,000xg for 10min at 4°C. Proteins were resolved by 10% SDS-PAGE, transferred to PVDF membranes and detected using the antibodies described above. Protein-antibody interactions were visualized using an ECF substrate and a Storm™860 (Molecular Dynamics) Scanning Instrument, and analyzed using Image Quant 5.0 software.

RNA isolation and cDNA synthesis

Total RNA was isolated with Trizol Reagent (RAW 264.7 cells) or RNeasy Mini Kit (peritoneal macrophages) according to the manufacturer's instructions. cDNA was generated from total RNA using Superscript II reverse transcriptase and random hexamers.

Reverse transcription (RT)-PCR

cDNA from RAW264.7 cells was used as a template for RT-PCR analysis using primers specific for TNFα (forward, 5'-tagccaggagggagaacaga-3', reverse, 5'- cacttggtggtttgctacga-3') or β-actin (forward, 5'-tatggagaagatttggcacc-3', reverse, 5'-gtccagacgcaggatggcat-3'). PCR products were separated on 1.8% agarose gel in the presence of ethidium bromide. The specific bands for TNFα (436bp) and β-actin (300bp) were visualized under UV light.

Quantitative real-time PCR

qRT-PCR was performed using TaqMan gene expression assays for mouse TNFα (Mm00443258_m1) and GAPDH control (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were performed in triplicates in 96 well plates on an ABI Prism 7700 Sequence Detection System and analyzed with SDS 1.9.1 software (Applied Biosystems). Levels of TNFα mRNA were normalized to GAPDH mRNA.

ELISA

The levels of TNFα, IL-1β, and IL-6 were determined by ELISA in 50μl samples, according to the manufacturer's protocol. The absorbance was measured at 450nm on a Vmax kinetic microplate reader with *Softmax pro* software (Molecular Devices). The sample values were calculated using a standard curve (0 - 1,500 pg/ml) and expressed per 10^6 cells or per ml serum.

ASMase acitivity assay

RAW264.7 cells were incubated for 2 hours in the absence or presence of 25μM desipramine. Cells were harvested and after centrifugation the pellet was re-suspended in PBS. Cells were homogenized by sonication and 20μg of total protein was incubated in an assay mix (0.5M acetate buffer pH4.5, 3μM NBD-SM, 15mM NaF, 1mM β-mercaptoethanol, protease inhibitors (1:200)) for 3h at 37°C. The reaction was stopped by addition of 0.5ml methanol. Cell debris was pelleted by centrifugation for 10 minutes at 16,000x g. The supernatants were analyzed by high performance liquid chromatography (HPLC) on a reverse-phase column (Nova PAC, C18) using methanol: water: orthophosphoric acid (850: 150: 0.150 by volume) as a mobile phase at a rate 2 of ml/min. NBD-ceramide formation was quantified after calibration using NBD-ceramide as an external standard.

Statistical analysis

Significant changes in multiple comparisons of two independent variables (treatment and genotype) were determined by two-way ANOVA with Bonferoni post-test. To test for differences among two or more independent groups unpaired t-test and one-way ANOVA were also used. A *P*-value <0.05 was considered significant. Data are reported as mean ± s.e.m (or ± s.d). The star symbol indicates the significance of the main effect (treatment or genotype) and a pound symbol indicates the significance of the interaction effect in two-way ANOVA analysis.

C. Results

C.1. Studies in RAW264.7 cells

Characterization of LPS signaling in macrophage cell line RAW264.7

To test the response of RAW264.7 cells to LPS stimulation, the activation of cell signaling molecules in the TLR4 proximity was assessed. Upon stimulation of the receptor, the adaptor molecule IRAK-1 becomes phosphorylated and degraded and the rate of its degradation has been linked to the extent of MAP kinase activation [81]. Therefore, the degradation of IRAK-1 was first investigated. RAW264.7 cells were treated with 10ng/ml LPS for various times and the degradation of IRAK-1 was monitored by Western blotting. As anticipated, the levels of IRAK-1 gradually decreased for up to 90 min following stimulation (Fig. 2.2 A). Next, the activation of downstream targets JNK and ERK was also tested by Western blotting using antibodies against the phosphorylated forms of these MAP kinases. In untreated control cells pJNK and pERK were almost undetectable, but transient phosphorylation of both kinases was

demonstrated in response to LPS, with maximum response at 30-40 min after stimulation followed by a decrease (Fig. 2.2 B, C). Dose dependent phosphorylation of JNK and ERK to increasing concentrations of LPS revealed that at 30 min, 5-10ng/ml of LPS was sufficient to induce maximum response (Fig. 2.3 A, B). These data indicate that RAW 264.7 cells respond potently to stimulation with LPS.

Production and release of TNFα by LPS stimulated RAW264.7 cells

To understand the dynamics of TNFα production and secretion by RAW264.7, the levels of TNFα mRNA were measured by RT-PCR, while the levels of TNFα in cell lysates and conditioned medium were measured by Western blotting. LPS-induced production of TNFα mRNA was detectable as early as 1 hour after stimulation and was maintained for up to 6 hours (Fig. 2.4 A). At the protein level, the TNFα detected in the cell lysates corresponded to the 26kD precursor form, while in the medium a band with a molecular weight of 17kD was found and represented the mature sTNFα, which was additionally confirmed with the positive control recombinant TNFα (Fig. 2.4 B). The dynamics of TNFα production and cleavage was further followed. In RAW264.7 cells proTNFα was detected as early as 2 hours and remained elevated for at least 10 hours, although some decrease was observed after 6h (Fig. 2.4 C). The appearance of sTNFα in the medium followed closely the levels of proTNFα in the cells, showing a slight delay in secretion. sTNFα was first detectable at 4 hours and continued to accumulate for at least 6 more hours (Fig. 2.4 D). In agreement with previous studies, a 17kDa unmodified form of sTNFα was predominant in the medium, although bands with a higher molecular weight were also detected (Fig. 2.4 D). It has been suggested that the majority of the proteins in the “ladder” are N-glycosylated forms of the 17kD sTNFα [77].

C.2. Studies in primary peritoneal macrophages

Induction of TNFα mRNA synthesis and sTNFα secretion in primary peritoneal macrophages after stimulation with LPS

Primary peritoneal macrophages were also tested as a more physiologically relevant cell culture model to study TNFα secretion in response to LPS. First, peritoneal macrophages were stimulated with 10ng/ml LPS and the levels of TNFα mRNA were measured by quantitative real-time PCR. Similarly to RAW264.7, in primary macrophages LPS induced robust synthesis of TNFα mRNA, which at its peak levels (4 hours) was more than a 100 fold higher compared to the vehicle treated control (Fig. 2.5 A). This induction, however was transient and at 6 hours post LPS a decrease in TNFα mRNA abundance was apparent.

The secretion of TNFα by activated macrophages was monitored by Western blotting. As expected, sTNFα accumulated in the medium with a similar kinetic as observed in RAW264.7 cells: it was detectable at 4 hours and increased at the later time points (Fig. 2.5 C). Furthermore, there was neither difference in the molecular size of the major 17kDa unmodified form nor in the glycosylation pattern between both cell types. However proTNFα in cell lysates from peritoneal macrophages was almost undetectable even though the amount of total protein which was loaded was the same as in RAW cells (Fig. 2.5 B). These results may indicate that despite a strong activation of LPS signaling and TNFα synthesis in both cell types, there might be a significant difference in post-translational processing of proTNFα.

C.3. Studies in mice

Dynamics of cytokine production in response to LPS *in vivo*

To develop a model to study TNFα production *in vivo*, mice were injected with saline or sub-lethal dose of LPS (5.8mg/kg) and the serum levels of TNFα, IL-1β, and IL-6 were measured by ELISA. The administration of LPS led to mild

and temporary inflammatory reaction in the host. A slight elevation in body temperature and lack of tonus were observed within a couple of hours. In all animals, these symptoms disappeared after approximately 6 hours. Control animals had undetectable levels of TNFα and IL-1β, but IL-6 ranged from 2 to 4ng/ml (Fig. 2.6 C, 0 time LPS). However, i.p. administration of LPS quickly induced a sharp but transient increase in the circulating levels of all three cytokines. Among them, the increase in TNFα secretion was the most rapid and dramatic, reaching 2.5ng/ml (more than 2000 fold increase over control animals) 1 hour after LPS injection (Fig. 2.6 A). The maximum levels of IL-1β and IL-6 were detected at 2h post stimulation (Fig. 2.6 B, C). These data are in agreement with previously published results and underlines the significance of TNFα as one of the earliest and most potent pro-inflammatory cytokines secreted during sepsis.

C.4. ASMase-deficient cells and animals as a model to study the role of ASMase in the LPS-induced TNFα synthesis

To study the role of ASMase and ASMase-derived ceramide in the regulation of TNFα production different *in vivo* and *in vitro* models were developed.

C.4.1. Inhibition of ASMase in RAW264.7 cells

To generate ASMase deficiency in RAW264.7 cells, a pharmacological inhibition of ASMase activity was first used. Desipramine is a cationic amphiphilic drug, which accumulates in the acidic compartments of living cells, since it is trapped as membrane-inpermeable form subsequent to its protonation in the acidic environment. This tricyclic amine decreases the binding of ASMase to its membrane-bound lipid substrate, which exposes the enzyme to lysosomal proteases and make it susceptible to proteolytic degradation [79]. Desipramine is widely used to inhibit ASMase activity, although some effects on acid ceramidase

have been also reported [80]. Treatment with 25μM desipramine was sufficient to inhibit more than 90% of ASMase activity in RAW264.7 cells when it was applied for 2 hours (Fig. 2.7 A). Importantly, the activity of Neutral sphingomyelinase (NSMase) in the same cells was not influenced by desipramine (Fig. 2.7 B). NSMase is the other signaling enzyme, which upon activation hydrolyzes sphingomyelin to ceramide; therefore, this result suggests that desipramine inhibits only the generation of ceramide by ASMase but not NSMase.

siRNA-mediated silencing was also tested as an alternative approach to suppress ASMase activity in RAW264.7 cells. Using fluorescently labeled siRNA (non-silencing siRNA-AlexaFluor555) the transfection conditions were first optimized to ensure maximal transfection efficiency. More than 70% of the cells contained siRNA based on the immunofluorescence (Fig. 2.8 A,B). Then, two different ASMase-specific siRNAs were used to knockdown ASMase. However, 24 and 48 hours after transfection the activity of ASMase was not different between the cells transfected with either of the silencing siRNAs and scrambled siRNA. A maximum of 10% inhibition was achieved at 72 hours after trasfection but it was still not significant compared to control cells (Fig. 2.8 C). Therefore, inhibition by desipramine but not silencing of ASMase by siRNA was used in further studies to inhibit ASMase in RAW264 cells.

C.4.2. Testing of ASMase-deficient fibroblasts, acquired from Niemann-Pick Disease patients and control healthy individuals, as a model to study the role of ASMase in TNFα production in response to LPS

Niemann-Pick disease (NPD) belongs to lysosomal storage diseases and it is characterized by a lack of ASMase activity. Mutations in the ASMase gene cause an abnormal accumulation of sphingomyelin, and in patients with the more severe form of the disease (type A) this affects the function of visceral organs and brain, and leads to lethality at an early age. Fibroblasts from patients with NPDA are often used to study the role of ASMase in various cellular responses.

Therefore, we tested fibroblasts from individuals with NPDA and healthy controls with intact ASMase activity as a model for deciphering the role of ASMase in TNFα production. After confirming the absence of ASMase activity in firbroblasts from NPDA patients (Fig. 2.9 A), control fibroblasts were treated with increasing concentrations of LPS and the levels of proTNFα in cells and sTNFα in medium were monitored by Western blotting. However, TNFα was not detectable either in the cells or in the medium even after treatment with 1μg/ml LPS, which is a concentration 100 fold higher compared to that used for treatment of macrophages (Fig. 2.9 B, C). Raw264.7 cells were used as a positive control in these experiments. Thus, these results demonstrated that fibroblasts were not an appropriate system to study TNFα secretion.

C.4.3. ASMase-deficient mice and macrophages as a model to study the role of ASMase in LPS-induced TNFα production

$Asm^{-/-}$ mice are the animal model of the human genetic disease NPDA. Therefore, $asm^{-/-}$ and litter-matched $asm^{+/+}$ (Fig. 2.10) mice, as well as peritoneal macrophages isolated from these mice, are a physiologically relevant model to understand the role of ASMase in TNFα production in response to LPS.

D. Discussion

The data presented in this chapter demonstrated that macrophages responded rapidly and strongly to LPS stimulation since the maximum activation of MAP kinases, ERK and JNK, was achieved 30-40 min following stimulation and as little as 1ng/ml of LPS was able to induce their phosphorylation. As discussed earlier the activation of MAPK has been strongly correlated with TNFα production. Consistent with these findings, LPS induced potent secretion of TNFα in both isolated peritoneal macrophages and the macrophage cell line, RAW264.7. Importantly, the time course for detection of TNFα in the medium as well as the molecular weight of TNFα were essentially the same for both cell

types. Although the 17kD unmodified form was the most abundant, additional bands with higher molecular weight were also detectable when substantial amount of medium was used for Western blotting. This pattern of “protein ladder” is consistent with previous studies demonstrating that the higher molecular bands represent TNFα glycosylated at different positions [77]. The physiological significance of this modification is currently unknown. However it has been shown that it does not influence the activity of sTNFα [78].

RAW 264.7 cells and primary peritoneal macrophages appeared to be similar in terms of sTNFα secreted in the medium, however there was a striking difference in the levels of proTNFα detected in cell lysates. Despite equal amounts of total protein used for Western blotting, proTNFα was almost undetectable in primary cells but abundant in RAW264.7 cells. These results indicate that there might be cell specific differences in the post-translational processing of proTNFα by RAW 264.7 cells and peritoneal macrophages.

Differences were also apparent between LPS-induced TNFα secretion *in vivo* and *in vitro*, since the stimulation of TNFα was much more rapid *in vivo* than *in vitro* - systemic TNFα peaked at 1 hour, while the earliest time point at which TNFα was detected in the medium was 4 hours. Additionally, the increase in TNFα levels was much more transient *in vivo* than *in vitro*; TNFα in the serum reached almost basal levels 6 hours after LPS administration, while in the medium it was readily detectable for more than 10 hours. Although the exact mechanisms for these differences are not precisely defined, there are several plausible explanations: Since the levels of TNFα mRNA started to decline after 4 hours, the accumulation of TNFα in the medium is most likely due to the lack of effective clearance mechanisms *in vitro*. In the circulation TNFα is rapidly cleared by the kidneys and liver, with estimated half-life of 6 to 20 min [82]. The delayed secretion of TNFα *in vitro* also can be due to the absence of factors, such as LBP, which have co-stimulatory effect *in vivo,* and facilitate the binding of LPS to the receptor and initiation of signal transduction. This possibility is further

supported by findings reported by Wollenberg et al. [83], that not only sTNFα but also TNFα mRNA reaches its peak levels much faster *in vivo* than *in vitro*.

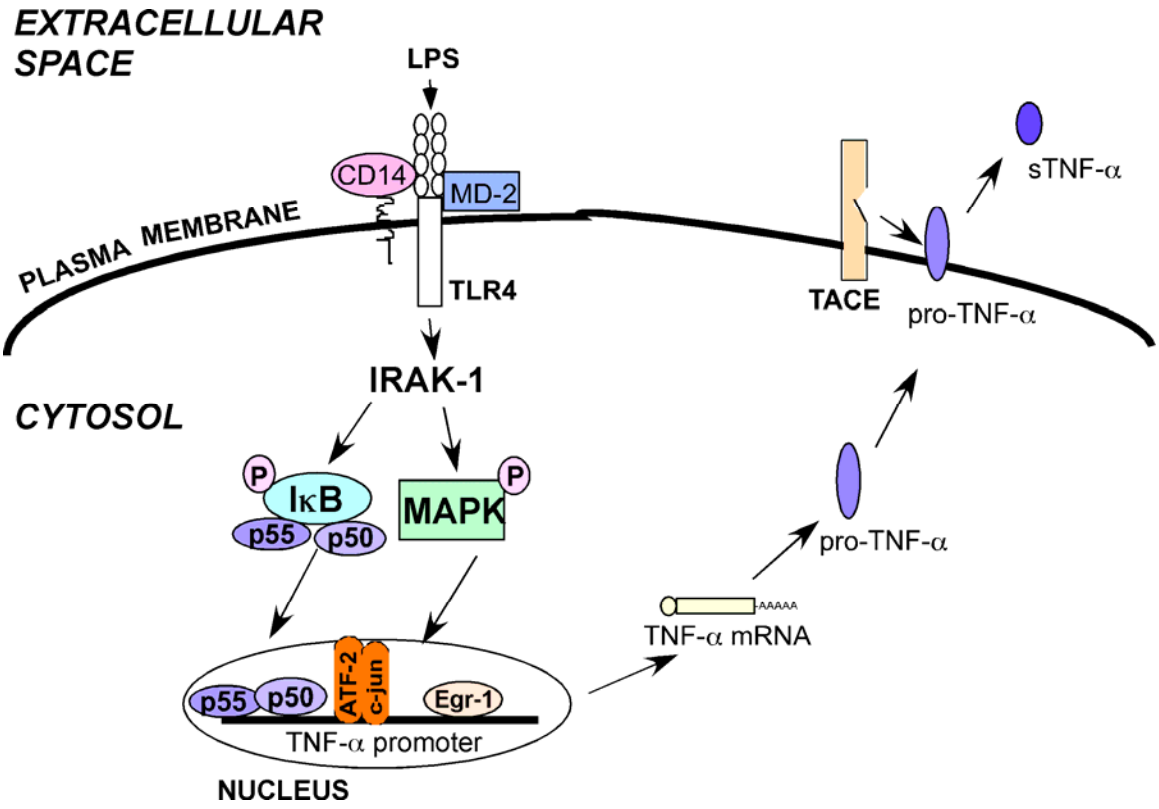


Figure 2.1. TNFα synthesis and secretion in response to LPS stimulation. The binding of LPS to the TLR4 signaling receptor induces phosphorylation and degradation of the adaptor molecule IRAK-1, which is essential for activation of downstream targets including MAP kinases. Phosphorylation of JNK, ERK, and p38, as well as degradation of Iκ-B, ultimately lead to nuclear translocation of transcription factors such as NF-κB, Elk-1, Egr-1, ATF-3, which are implicated in the initiation of TNFα gene transcription. LPS stimulation increases the stability of TNFα mRNA, which is translated into 26kD TNFα precursor (proTNFα). proTNFα is transferred to the plasma membrane, where it is cleaved by TACE to produce the 17kD soluble biologically active TNFα (sTNFα).

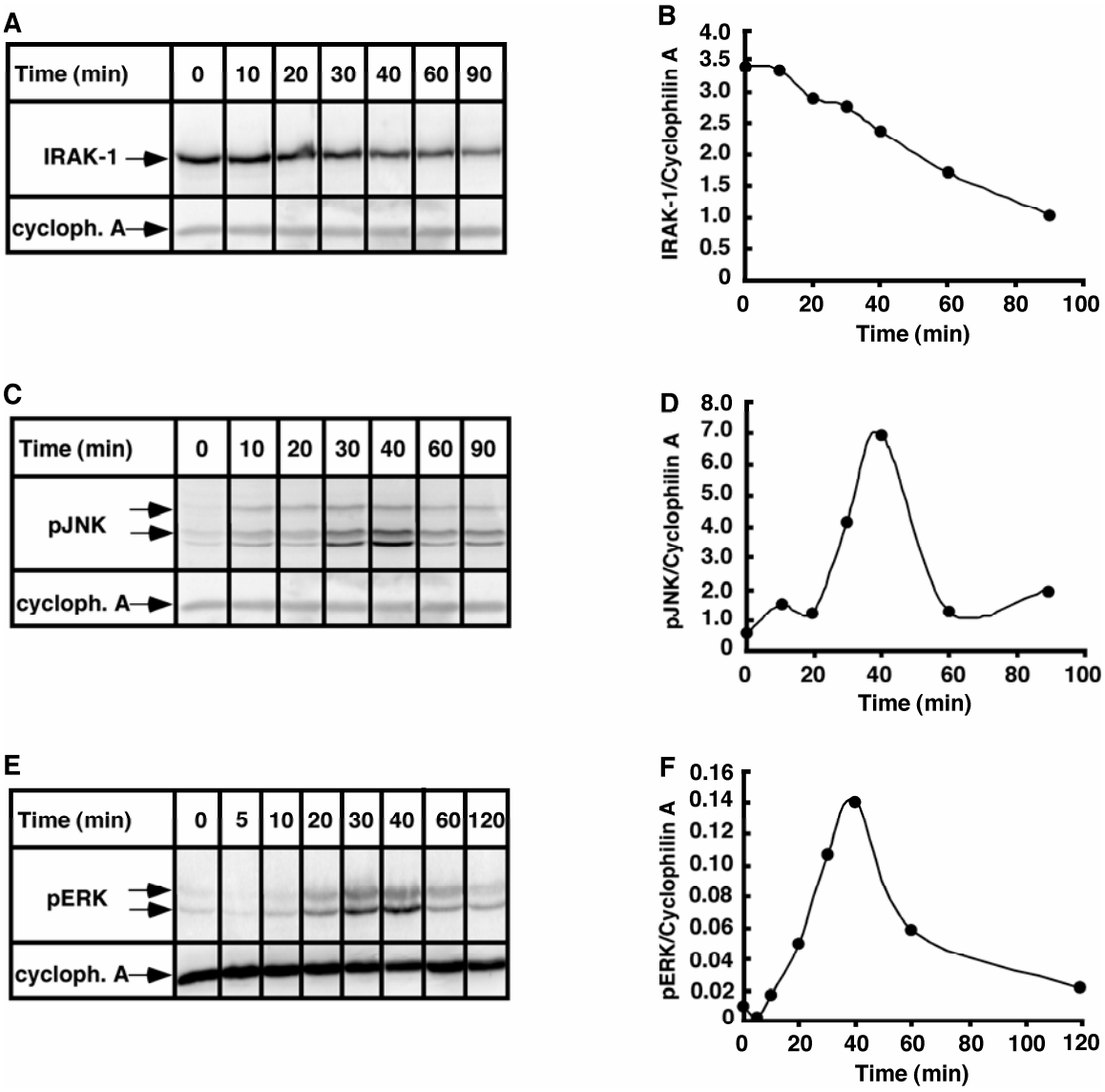


Figure 2.2. Time course of IRAK-1 degradation and phosphorylation of ERK and JNK in RAW264.7 cells stimulated with LPS. RAW264.7 cells were treated with LPS (10ng/ml) for the indicated time. The rate of IRAK-1 degradation (A, B) and phosphorylation of JNK (C, D) and ERK (E, F) was detected by Western blotting using specific antibodies. Cyclophilin A was used to ensure equal protein loading. Representative image is shown and the quantification of the band intensity for IRAK-1, pJNK, and pERK is presented normalized to the levels of cyclophilin A in the same sample (B, C, D)

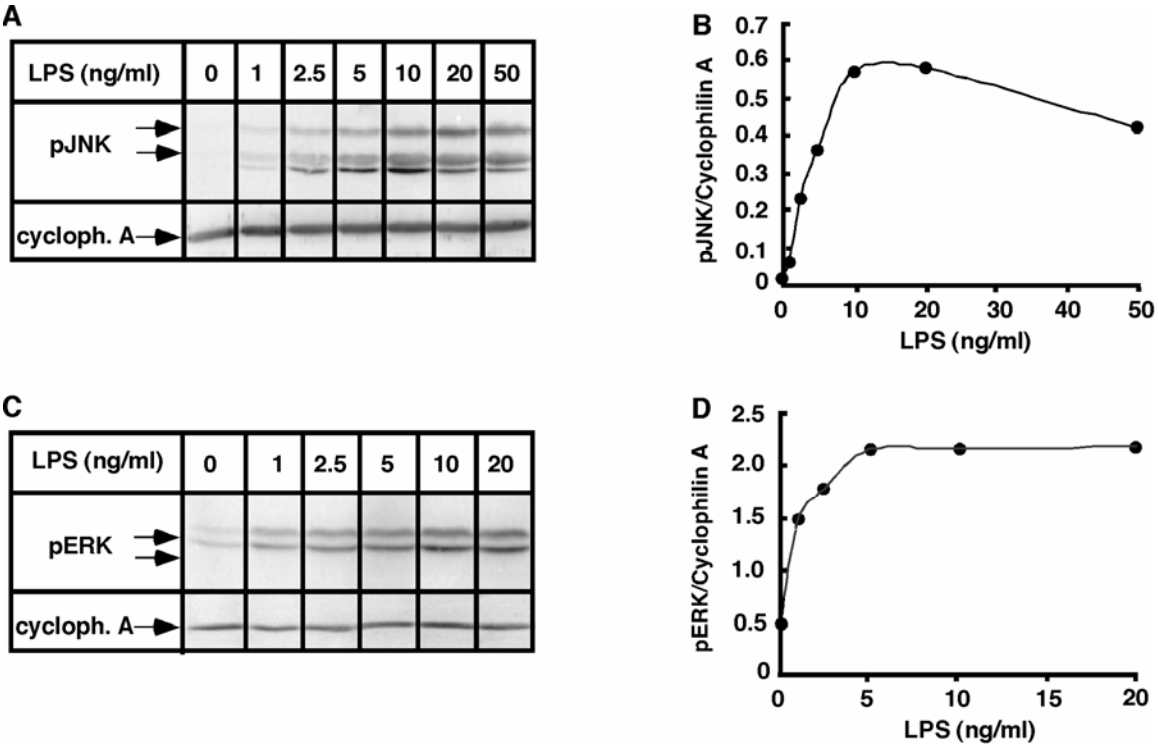


Figure 2.3. Dose dependent phosphorylation of ERK and JNK after LPS treatment. RAW264.7 cells were treated for 30min with increasing concentrations of LPS. Phosphorylation of JNK (A, B) and ERK (C, D) was determined by Western blotting using antibodies specific for the phosphorylated forms of JNK and ERK. Cyclophilin A was used to control for uniform loading. Representative image is shown and the quantification of the band intensity for pJNK, and pERK is presented after normalization to the levels of cyclophilin A in the same sample (B, D).

RAW264.7 cells

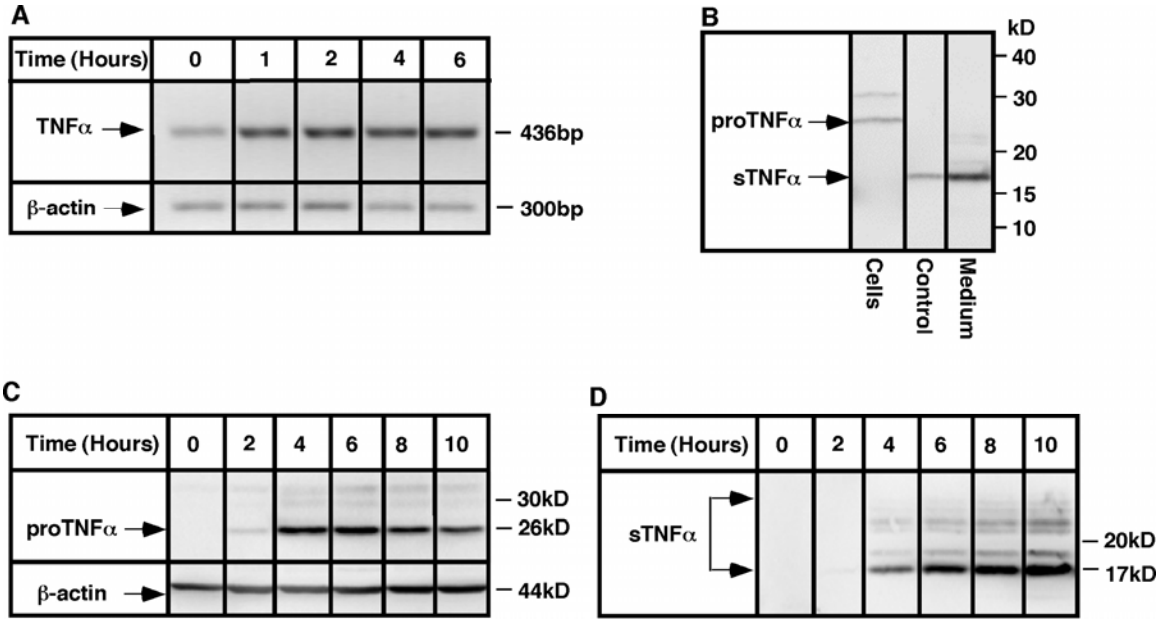


Figure 2.4. TNFα mRNA levels and post-translational processing of TNFα precursor in RAW264.7 cells. RAW264.7 cells were treated with LPS (10ng/ml) for the indicated times. **A. TNFα mRNA levels.** Total RNA was isolated by Trizol reagent and the levels of TNFα mRNA were determined by RT-PCR. The expression level of β-actin mRNA was used to control for uniform loading (A). **B-D. TNFα processing.** The kinetics of 26kD proTNFα processing to 17kD sTNFα was determined by Western blotting. Recombinant human TNFα was used as a positive control for sTNFα (B). Time course of proTNFα production in cell lysates (C) and its cleavage to sTNFα measured in the medium (D).

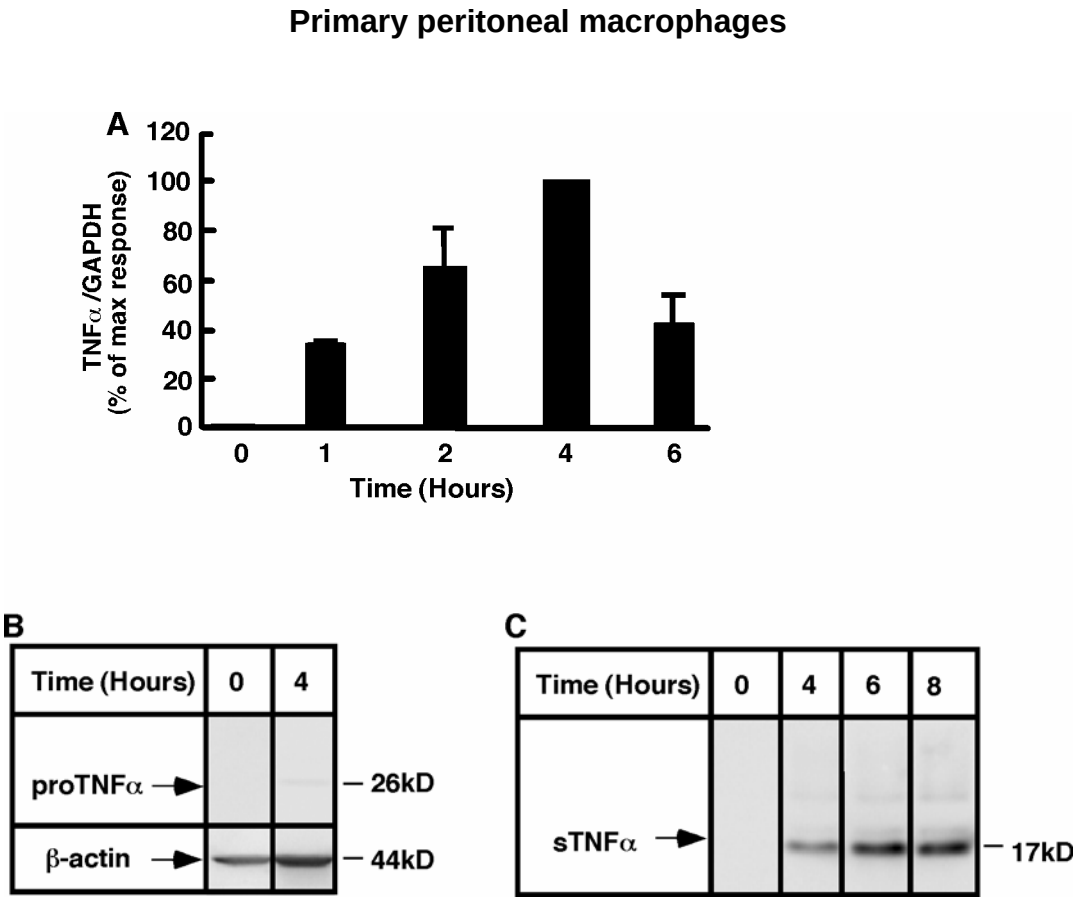


Figure 2.5. TNFα mRNA levels and post-translational processing of TNFα in primary peritoneal macrophages. Peritoneal macrophages were isolated from C57BL/6 mice and treated with LPS (10ng/ml) for the indicated times. **A. TNFα mRNA expression.** The levels of TNFα mRNA in each sample were measured in triplicates by quantitative real-time PCR and normalized to GAPDH levels in the same sample. The results from two independent experiments are shown and expressed as percent of the maximum response (A). **B and C. TNFα processing.** The levels of proTNFα in cell lysates (B) and sTNFα in the medium (C) were determined by Western Blotting. β-actin levels were used to control for uniform loading.

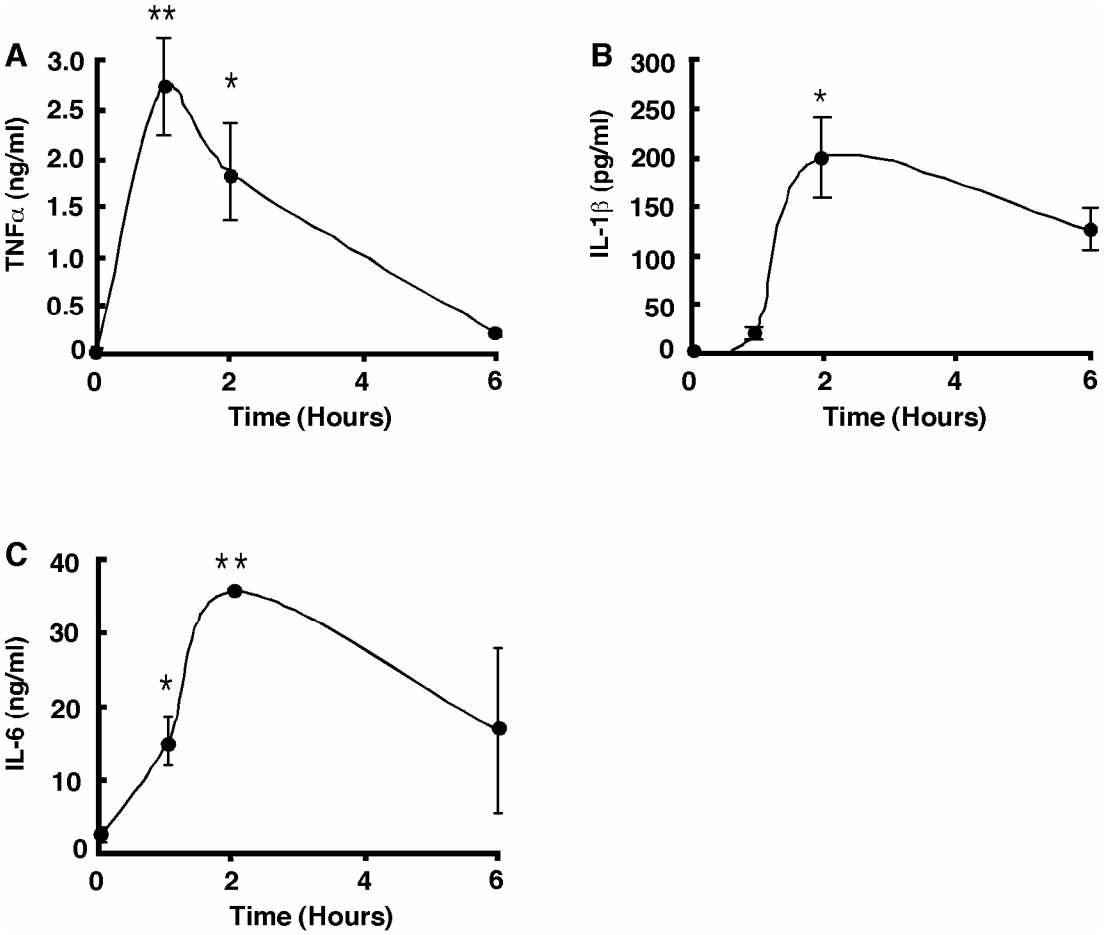


Figure 2.6. Cytokine levels in LPS-injected C57BL/6 mice.

C57BL/6 mice were injected i.p. with LPS (5.8mg/kg body weight) or saline. Serum was collected at the indicated times and the levels of TNFα (A), IL-1β (B), and IL-6 (C) were measured by ELISA. Only the data for LPS-injected animals are shown. The levels of the cytokines in saline-injected animals were below the detection levels for TNFα and IL-1β and were around 2ng/ml for IL-6 (C). The combined data from three independent experiments are shown as group average ± s.e.m. The significance of the LPS treatment effect (**, $P<0.01$, *, $P<0.05$) is based on one-way ANOVA.

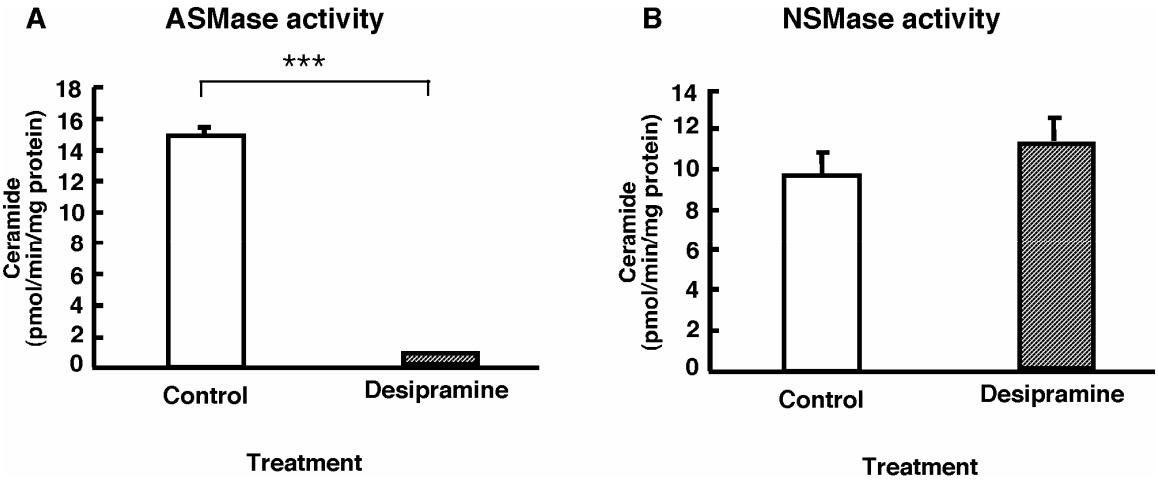


Figure 2.7. Effect of desipramine on ASMase and NSMase activity in RAW264. 7 cells. RAW264.7 cells were treated with desipramine (25μM) for 2 hours. ASMase (A) and NSMase (B) activities were measured in cell lysates by using NBD-sphingomyelin as a substrate and HPLC method. Statistical significance of desipramine effect is shown (***<0.001) based on unpaired student t-test.

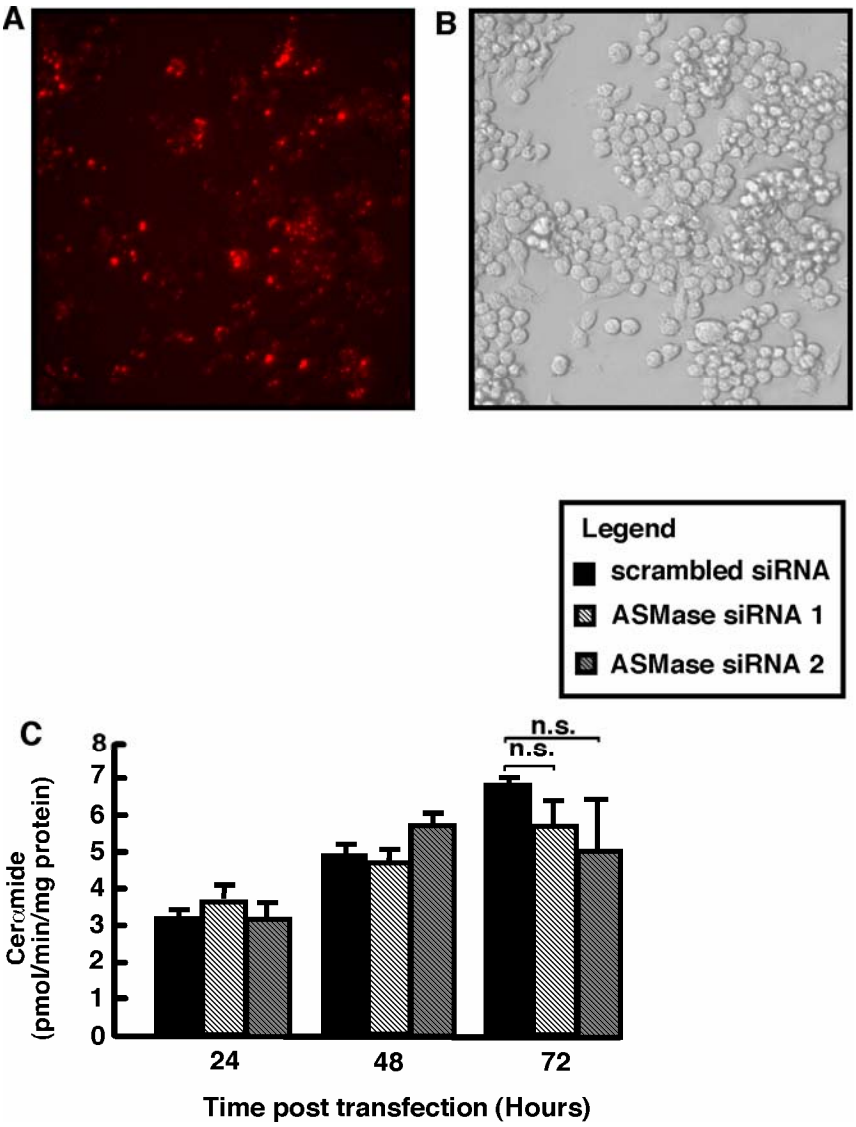


Figure 2.8. ASMase silencing by siRNA in RAW264.7 cells. RAW264.7 cells were transfected with either scrambled or ASMase specific siRNA. Transfection efficiency at 6 hours is shown after using scrambled siRNA labeled with Alexa Fluor 555 and fluorescent microscopy (A). Transmitted light images of RAW264.7 cells (B). ASMase activity was measured in cell lysates from RAW264.7 cells transfected for 24, 48, or 72 hours with control or ASMase specific siRNA. Two different ASMase specific siRNAs were used designated as ASMase siRNA 1 and ASMase siRNA 2 (C).

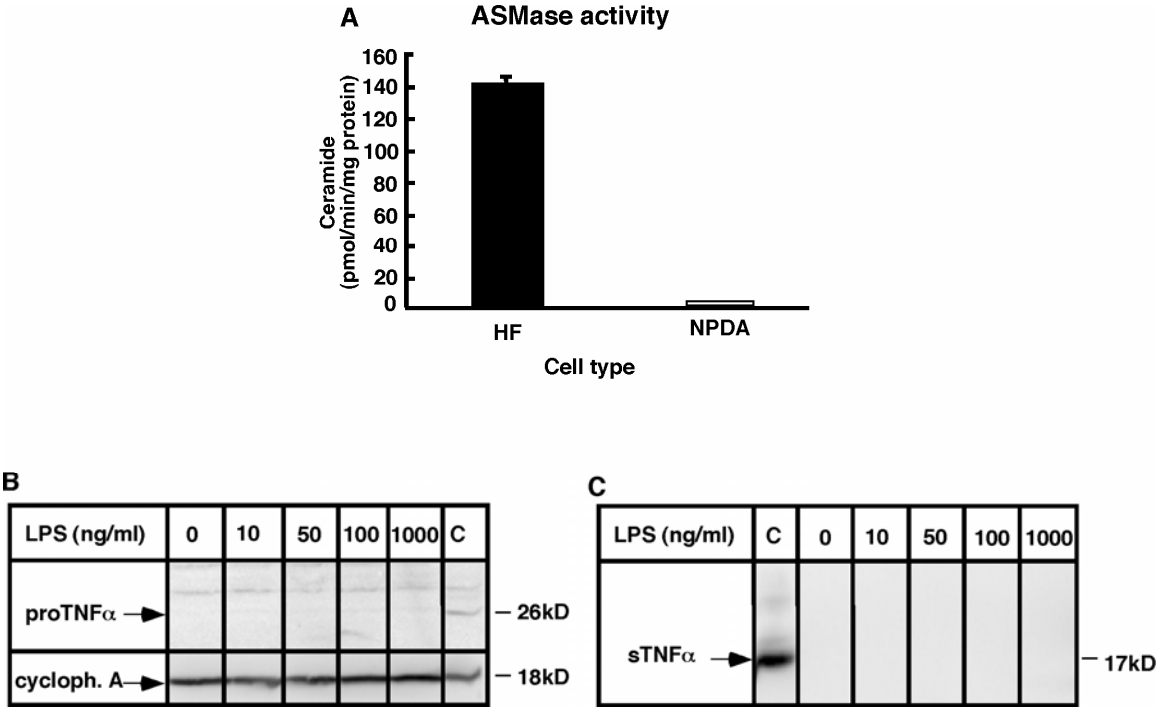


Figure 2.9. Characterization of LPS response in human fibroblasts. A. ASMase activity. ASMase activity in fibroblasts from healthy individuals or patients with type A Niemann-Pick Disease measured by the HPLC method (A). **B and C. TNFα levels.** Human skin fibroblasts from healthy individuals were treated for 4 hours with increasing concentrations of LPS and the levels of proTNFα in cell lysates (B) and sTNFα in medium were determined by Western blotting. Cell lysate and medium from LPS stimulated RAW264.7 cells were used as a positive control.

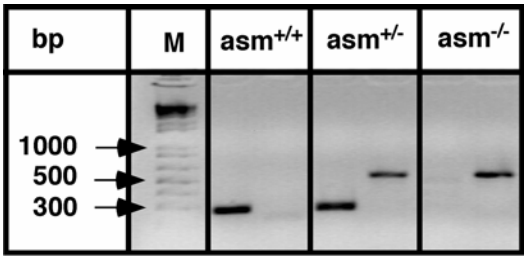


Figure 2.10. ASMase-deficient mice - an *in vivo* model to study the role of ASMase in TNFα secretion. The offspring of heterozygous ($asm^{+/-}$) breeding pairs were genotyped after weaning by using PCR of tail DNA. Around 25% of the mice were $asm^{-/-}$ and had single PCR product of 550bp. Litter-matched $asm^{+/+}$ controls were also around 25% of the offspring and had single PCR product of 300bp. In the heterozygous mice two bands corresponding to 550bp and 300bp were detectable.

CHAPTER THREE

Role of ASMase and ceramide in the transcriptional regulation of TNFα synthesis in response to LPS

A. Introduction

LPS is one of the most potent agonists that activates mononuclear cells to produce TNFα. The recognition of LPS by macrophages is mediated by LBP, which binds to the lipid A moiety [84] and greatly increases host sensitivity to LPS [85]. LBP-LPS forms a ternary complex with CD14, which enables LPS to be transferred to the signal transducing receptor complex. CD14 exists in two forms: a soluble CD14, which is found in the plasma; and membrane-bound CD14, which is attached to the surface of myeloid cells via a glycosylphosphatidylinositol anchor. The LPS-specific receptor complex is composed of Toll-like receptor-4 (TLR4) and MD-2. MD-2 is secreted glycoprotein and acts as an extracellular adaptor protein in the activation of TLR4. MD-2 is important for ligand recognition by TLR4 and is essential for LPS signaling to occur. Mice lacking MD-2 are unresponsive to LPS [86]. TLR4 is the first-discovered mammalian homologue of Drosophila Toll. TLR4 works downstream of CD14 and is responsible for delivering an LPS signal. Homodimerization of TLR4 recruits to the receptor complex adaptor proteins Mal and MyD88, which, analogously to the IL-1β signaling, associate with IRAK-1 causing its phosphorylation. Phosphorylation of IRAK-1 is followed by its ubiquitination and proteosome-mediated degradation, which determines the magnitude of TLR response. For example, inhibition of IRAK-1 degradation in macrophages by inhibitors of ubiquitin ligases has been found to potentiate the inflammatory response, as judged by a more potent induction of JNK [87]. In turn, decreased stability of IRAK-1 underlies the decreased response to a second dose of LPS and is the cause of endotoxin tolerance [88].

Hyperphosphorylation of IRAK-1 is also important for dissociation from the receptor complex and association of IRAK with TRAF6, which is critical for the activation of MAPK kinase TAK-1. TAK-1 acts as a common activator of NF-κB as well as of p38 and JNK. The activation of MEK/ERK1/2 pathway in response to LPS appears to be Raf-1 dependent.

Most of the above mentioned signaling molecules have been shown to be essential for TNFα production and are implicated in various mechanisms of its regulation. For example, stimulation of LBP production by LPS [89] has been linked to an increase in TNFα mRNA synthesis and stability [90]. The requirement of CD14 for TNFα synthesis was reinforced by the inhibition of TNFα production in the presence of blocking antibodies to CD14 [91]. Additionally, a number of studies unequivocally demonstrated the importance of MAPK and NF-κB for LPS induced TNFα production. For example, dominant-negative forms of both Rac and c-Raf inhibited LPS induction of TNFα promoter in RAW264.7 cells [62]. It has also been found that treatment of cells with ERK inhibitor, PD-098059, reduced TNFα mRNA expression in a dose dependent manner; and TNFα secretion was completely abolished in the presence of Ro 09-2210, a MEK inhibitor which blocks the activation of not only ERK1/2, but also JNK and p38 [73]. Additional studies further supported the role of p38 activation for TNFα production [74], and also suggested that JNK and p38 might be involved in the translational regulation of TNFα [75, 92, 93].

The enhancement of transcription initiation, release of the transcript elongation block, and transcript stabilization contribute to the accumulation of TNFα mRNA in stimulated macrophages [94, 95]. Transcription factors such as c-Jun, ATF-2, Egr-1, and Elk-1 [96], which are downstream targets of MAPK have been shown to be essential for the initiation of transcription and RNA synthesis. Many of the above mentioned factors bind to the proximal promoter region, which plays an important functional role in the transcriptional regulation of mouse TNFα [97]. Chromatin immunoprecipitation revealed that ATF-2, c-Jun, Elk-1, and Ets-1 bind to this region in J774 cells [98]. 5’ distal NF-κB sites are also important for maximum functional activity of mouse TNFα gene [68, 97, 99].

Finally, a mutation in the Egr-1 site in the TNFα promoter significantly reduced the level of LPS induction [67]. Once transcribed, TNFα mRNA is stabilized after LPS stimulation. Cis-acting elements in the TNFα 3'-untranslated region are sufficient for LPS-induced activation of TNFα translation [76, 100]. All three MAP kinases, ERK, JNK and p38 have been implicated in the regulation of TNFα mRNA stability [75, 101, 102].

The generation of ceramide in response to various stimuli has been linked to both up-regulation and down-regulation of NF-κB and MAPK activity in different cell types. In IL-1β stimulated primary hepatocytes, ceramide produced by NSMase led to an increase in JNK phosphorylation [81]. Activation of JNK by ASMase-derived ceramide has also been reported after stimulation with UV light [103], and after infection with *Pseudomonas aeruginosa* [104]. However, ceramide generated after infection with protozoan parasite Leishmania donovani inhibited ERK phosphorylation [105] and suppressed AP-1 and NF-κB activation [106]. Cell permeable C_2-ceramide was shown to stimulate ERK activation, which appeared to be important for smooth muscle contraction [107]. However, in RBL-2H3 cells C_2-ceramide suppressed phosphorylation of ERK and p38 [108]. Finally, ceramide stimulated p38 phosphorylation in HL-60 cells [109] but it was linked to p38 dephosphorylation in PMA stimulated human breast cancer cells [110].

Modulation of MAPK and NF-κB activity, which are essential for TNFα transcriptional and translational regulation by ceramide, suggests that it might play a role in TNFα production. The effects of ceramide, however, appear to depend on the stimulus, cell type, and pathway for its generation. Therefore, this chapter investigated the role of ASMase-derived ceramide in LPS-induced transcriptional regulation of TNFα production.

B. Materials and Methods

Materials

Bacterial sphingomyelinase (*S. Aureus*) was purchased from Sigma-Aldrich Co. (St. Luis, MO). C_2-ceramide was from Avanti Polar Lipids (Alabaster, AL).

Animals

A colony of ASMase *knockout* ($asm^{-/-}$) mice maintained in C57BL/6 background were propagated using heterozygous breeding pairs and genotyped by PCR of tail DNA. The mice received food and water *ad libitum* and were housed in AAALAC-approved animal facility of the University of Kentucky Medical Center. Litter-matched wild-type ($asm^{+/+}$) and $asm^{-/-}$ mice were used for experiments.

SDS-PAGE and Western blotting

Please refer to Chapter Two, Section B.

RNA isolation, Reverse transcription (RT)-PCR, and Quantitative real-time PCR analysis

Please refer to Chapter Two, Section B.

ELISA

The levels of TNFα in the medium from $asm^{+/+}$ and $asm^{-/-}$ miacrophages were determined by ELISA as described in Chapter one, Section B.

Statistical analysis

Please refer to Chapter Two, Section B.

C. Results

Genotype-specific differences in the level of TNFα secreted by macrophages stimulated with LPS

To test whether ASMase deficiency affected the production of TNFα, peritoneal macrophages were isolated from $asm^{+/+}$ and $asm^{-/-}$ animals and stimulated with LPS. The release of TNFα in the medium was monitored by ELISA. As anticipated, treatment with LPS induced TNFα secretion, which reached maximum at 4 hours after stimulation. However, TNFα levels were up to 2 fold higher in $asm^{-/-}$ macrophages as compared to $asm^{+/+}$ cells (Fig. 3.1 A), although the difference tended to decrease at later time points (6 hours). Furthermore, the effect of ASMase seems to be specific for TNFα secretion, since the levels of IL-1β were not significantly different between $asm^{+/+}$ and $asm^{-/-}$ macrophages (data not shown). ASMase deficient macrophages lack one of the major enzymes responsible for the generation of ceramide, therefore to demonstrate a direct link between ceramide and sTNFα levels, $asm^{-/-}$ were treated with cell permeable C_2-ceramide and TNFα levels were measured in the medium. The addition of short-chain ceramide to $asm^{-/-}$ macrophages inhibited sTNFα secretion in a dose dependent manner (Fig. 3.1 B). Together, these results indicate that ASMase and ceramide may regulate specific step(s) in TNFα production.

Effects of ASMase on TNFα levels are not mediated by PGE_2 or IL-6

The secretion of TNFα by LPS-activated macrophages can be modulated by a variety of pro- and anti-inflammatory mediators induced by LPS or TNFα itself. Therefore, it is important to understand whether ASMase directly affects LPS-induced TNFα production, or if it acts through secondary mechanisms. Since PGE_2 is one of the well-known negative regulators of TNFα secretion, its levels were measured in medium from $asm^{+/+}$ and $asm^{-/-}$ macrophages. PGE_2

secretion was induced after LPS stimulation and gradually increased for at least up to 8 hours. However $asm^{-/-}$ and control cells released similar levels of PGE_2 at all time points tested, suggesting that the differences in TNFα secretion are not attributable to a defect in PGE_2 production (Fig. 3.2 A). Similarly, the simultaneous treatment with IL-6, which can also suppress TNFα synthesis, had no effect on the genotype-related differences (Fig. 3.2 B).

ASMase deficiency has no effect on MAP kinase activation and TNFα mRNA production in peritoneal macrophages

To determine whether the observed differences in TNFα secretion are related to differences in the LPS signaling pathway at the plasma membrane, the activation pattern of proteins proximal to the LPS receptor was first examined. However, the IRAK-1 degradation rate was not different between genotypes (Fig. 3.3 A, B). Similarly, the pattern of ERK phosphorylation was not affected by the lack of ASMase activity (Fig. 3.3 C, D). Finally, the magnitude and time course of LPS-induced TNFα mRNA production was the same in both genotypes (Fig. 3.3 E). These results are in agreement and extend previous studies that examined the activation of MAP kinase pathway in macrophages from $asm^{-/-}$ and $asm^{+/+}$ mice, and found no differences in the ability of TNFα and LPS to induce activation of ERK, JNK, p38 and NF-κB [111].

Inhibition of ASMase by desipramine affects neither LPS-induced phosphorylation of ERK and JNK nor TNFα mRNA synthesis

The effect of ASMase on transcriptional regulation of TNFα was also tested in RAW264.7 cells, where acute inhibition of ASMase was achieved after treatment with desipramine. Although desipramine suppressed ASMase activity by more than 90% (See chapter two, Fig. 2.5 A), which was consistent with the results from primary macrophages, the extent of MAP kinase phosphoryaltion and induction of TNFα mRNA synthesis were similar among treatments and were

not influenced by ASMase deficiency (Fig. 3.4 A, B, C, D). Furthermore, the treatment of RAW264.7 cells with either C_2-ceramide or bacterial sphingomyelinase, both of which increased ceramide levels, did not have any effect on TNFα mRNA levels (Fig. 3.4 E). Altogether these results suggest that ASMase and ceramide do not affect transcriptional regulation of TNFα production.

D. Discussion

The activation of ASMase by LPS followed by the generation of ceramide have been previously reported and suggested to be important for the initiation of signal transduction and regulation of the activity of signaling molecules such as MAPK. The results presented in this chapter demonstrated that ASMase is involved in the regulation of TNFα production following LPS stimulation, and the lack of ASMase activity was linked to increased levels of sTNFα, suggesting that it plays a role of negative regulator of TNFα production. Additional treatments confirmed such a conclusion, demonstrating that exogenous ceramide is sufficient to decrease sTNFα in the medium. ASMase and ceramide appear to influence directly LPS-induced TNFα production, rather than working via secondary mechanisms. However, the activation of ERK and JNK as well as the levels of TNFα mRNA were not influenced by ASMase deficiency in neither primary macrophages nor RAW 264.7 cells, indicating that ASMase and ceramide most likely affect step(s) of TNFα production downstream of TNFα mRNA synthesis.

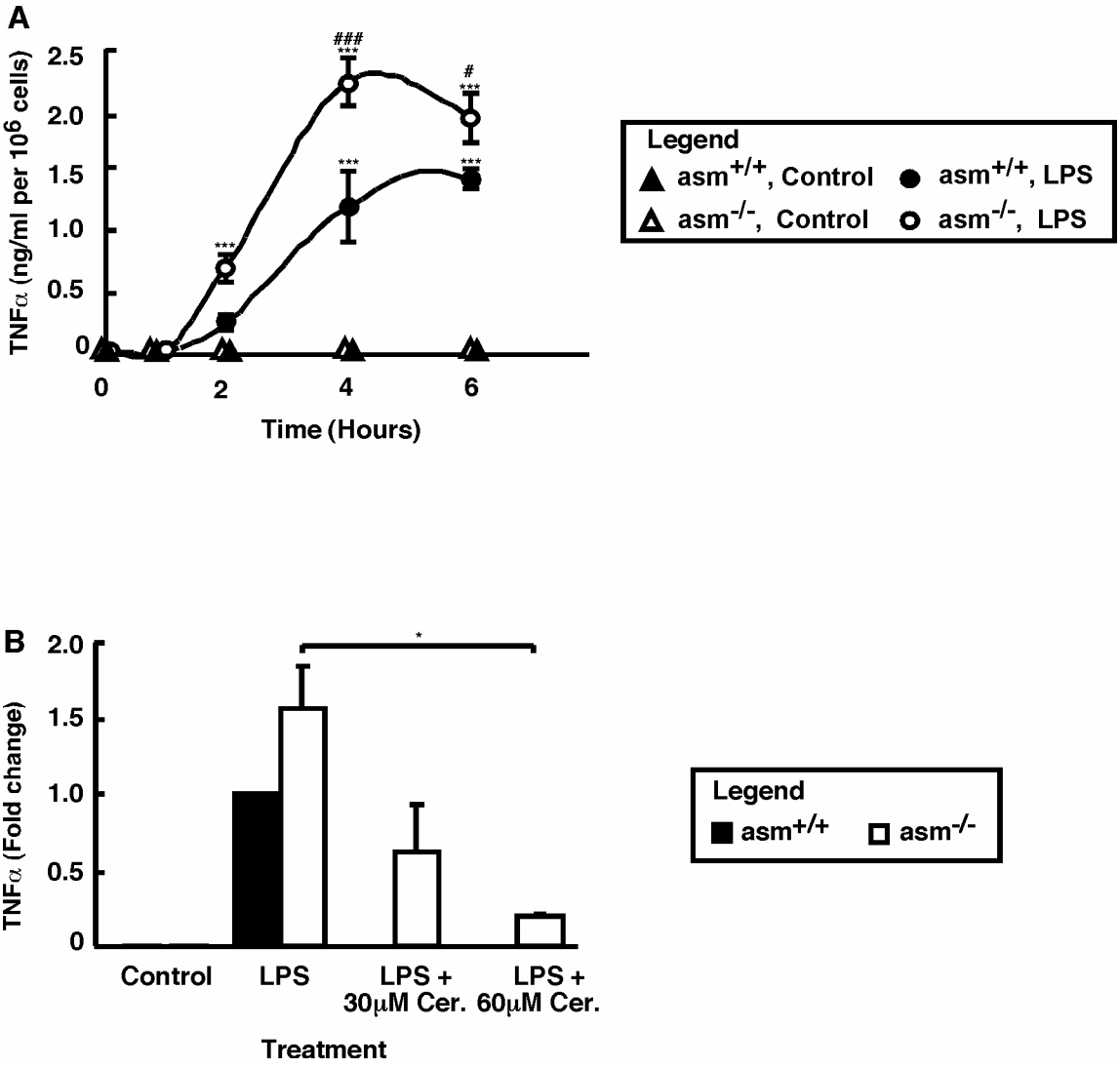


Figure 3.1. Effect of ASMase and ceramide on TNFα production in primary macrophages. TNFα levels measured by ELISA in medium of peritoneal macrophages from $asm^{+/+}$ (filled symbols) or $asm^{-/-}$ (open symbols). Cells treated with LPS (100ng/ml, circles) or PBS (triangles) for the indicated times. Representative results of three independent experiments are shown (A). Cells treated with LPS (10 ng/ml) for 4 hours in the presence of the indicated concentrations of C_2-ceramide or vehicle control (0.1% ethanol). Results combined from two independent experiments are shown (B). Data are presented as mean ± s.e.m. Statistical significance of the main effect (***, $P<0.001$) and the interaction effect (###, $P<0.001$; #, $P<0.05$) are shown based on two-way

ANOVA. The statistical significance of ceramide effects was calculated by one-way ANOVA (*, $P<0.05$).

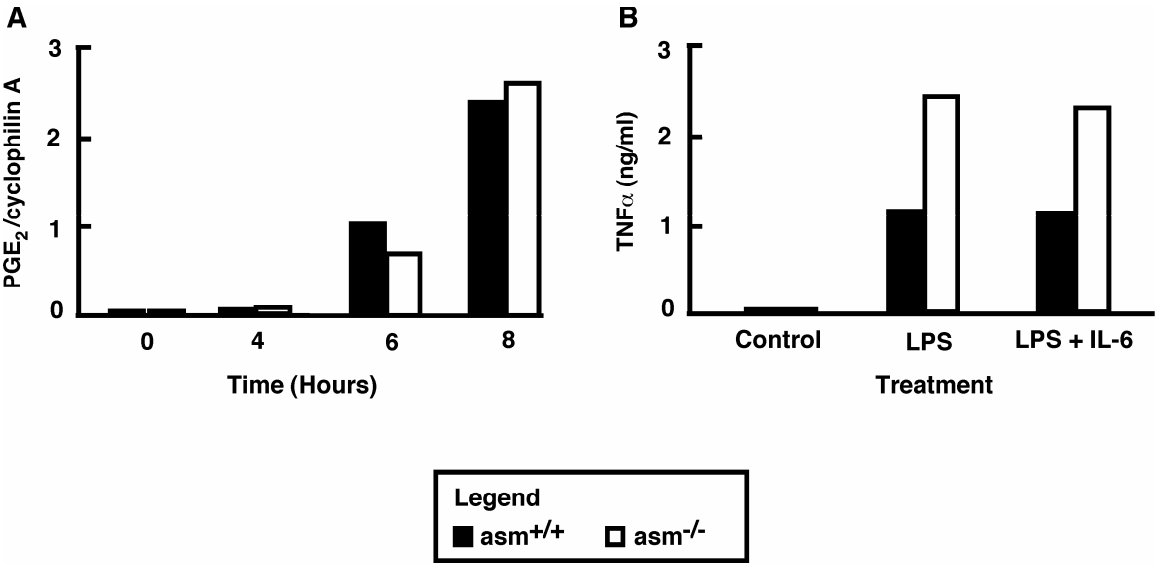


Figure 3.2. Role of ASMase deficiency on PGE_2 production and IL-6 effects on TNFα secretion. Peritoneal macrophages from $asm^{+/+}$ (filled bars) or $asm^{-/-}$ (open bars) were treated with LPS (10ng/ml) for the indicated time. The levels of PGE_2 in cell-free conditioned medium were determined by ELISA and the results were normalized to the cyclophilin A measured in the cells (A). Peritoneal macrophages from $asm^{+/+}$ or $asm^{-/-}$ were treated for 4 hours with LPS (10ng/ml) in the absence or presence of IL-6 (20ng/ml). The levels of TNFα (ng/ml) in the medium were measured by ELISA.

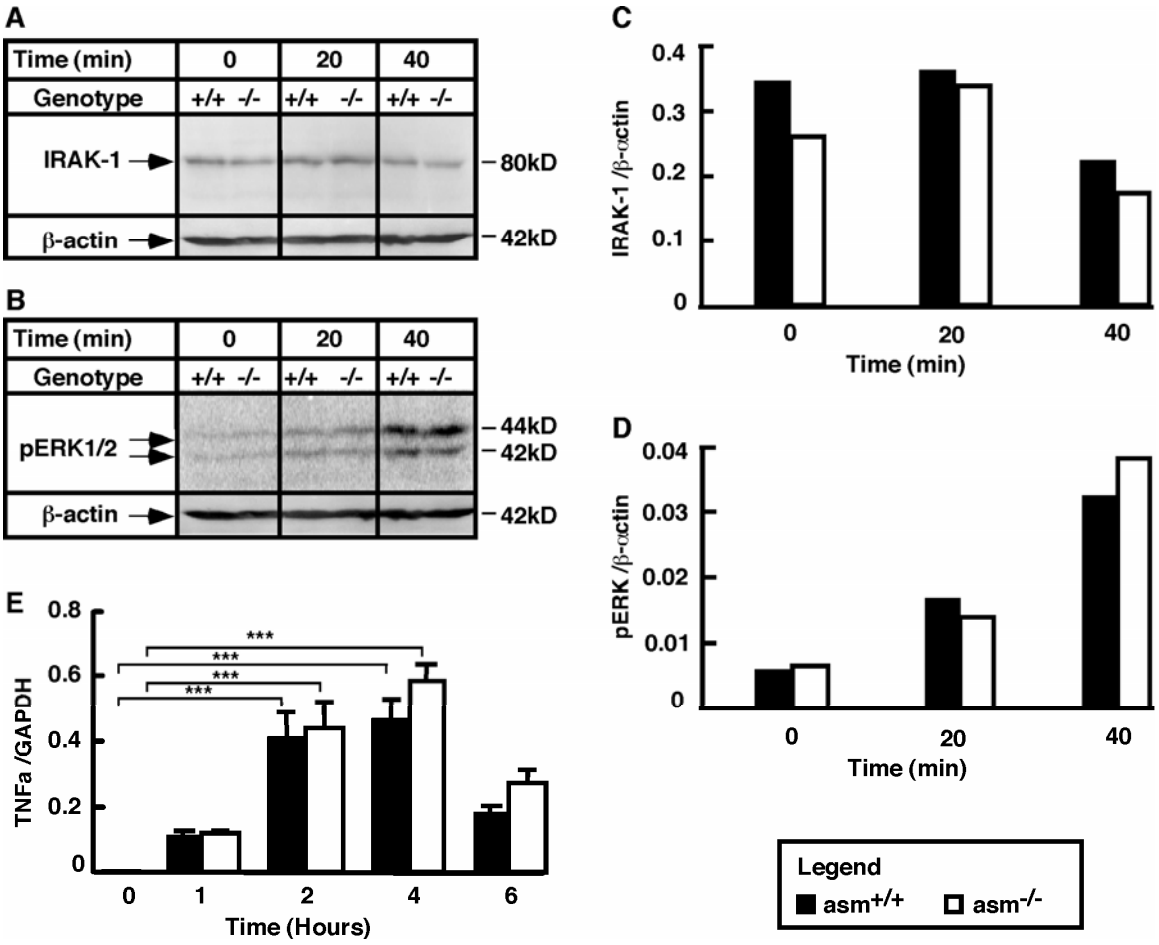


Figure 3.3. Stimulation of IRAK-1, ERK, and TNFα mRNA in asm$^{+/+}$ and asm$^{-/-}$ macrophages. Peritoneal macrophages from asm$^{+/+}$ (filled bars) and asm$^{-/-}$ (open bars) mice were treated with LPS (10ng/ml) for the indicated times. A-D. **Activation of IRAK-1 (A, C) and ERK1/2 (B, D).** Analyses were done using Western blotting and antibodies against IRAK-1 and phosphorylated ERK. Data are representative of three independent experiments. β-Actin levels were used to control for uniform loading. **(E) Stimulation of TNFα mRNA.** mRNA levels were determined by real-time PCR. GAPDH mRNA level was used for normalization. Data are presented as mean ± s.e.m. of three independent experiments. Statistical significance of the treatment effect is shown (***, P<0.001) based on two-way ANOVA.

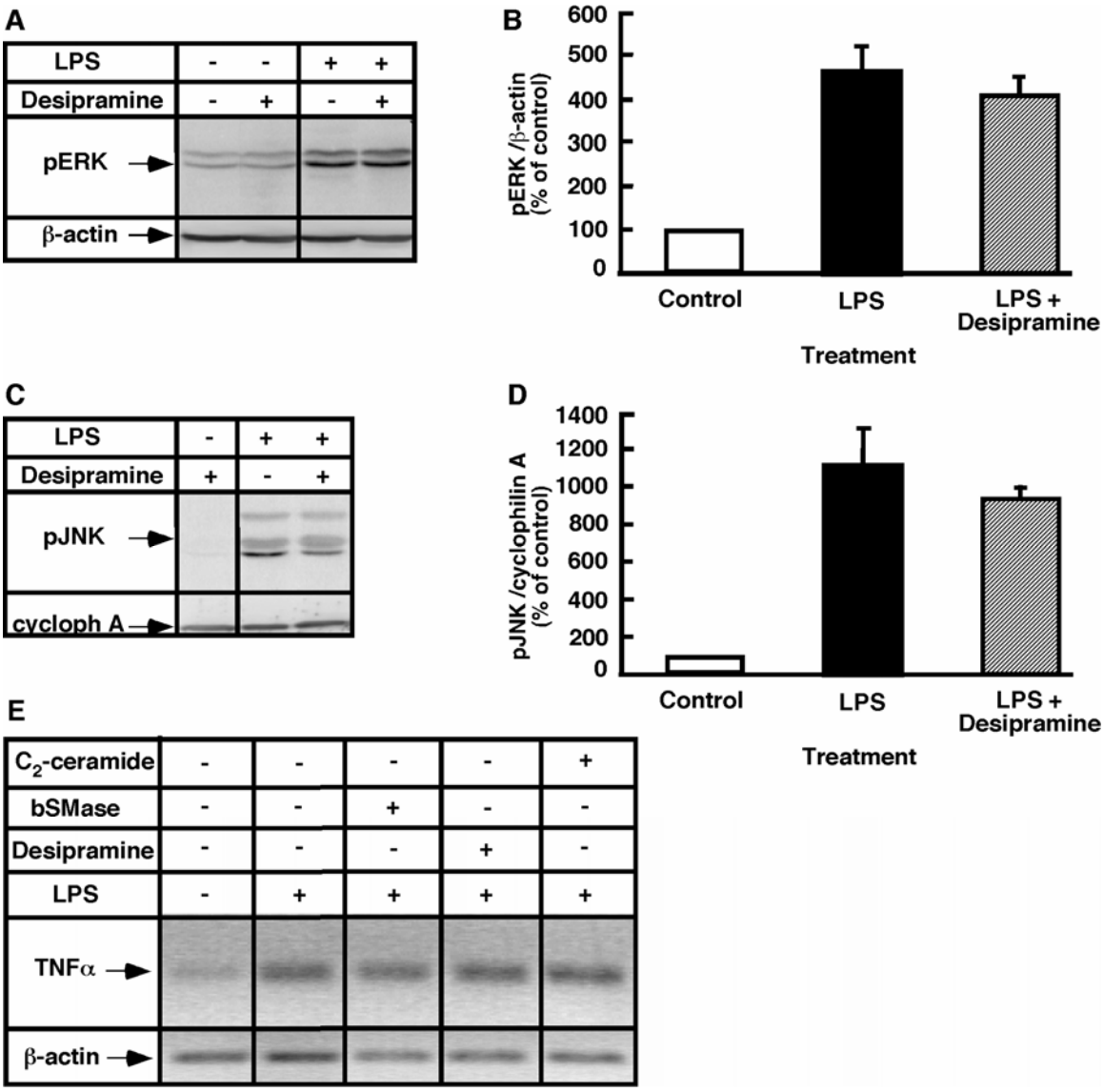


Figure 3.4. Phosphorylation of ERK and JNK, and production of TNFα mRNA in LPS stimulated ASMase deficient and control RAW264.7 cells. A-D. Effect of ASMase deficiency on the LPS-induced activation of ERK and JNK. RAW264.7 pre-treated with desipramine (25μM) to inhibit ASMase activity and control cells were stimulated with LPS (10ng/ml) for 60 min. Phosphorylation pattern of ERK (A) and JNK (C) after LPS stimulation was determined by Western blotting using antibodies specific for the phosphorylated forms of these MAP kinases. Quantification of pERK and pJNK band intensity is shown after normalization to the cyclophilin A (B,D), used as a control for equal protein

loading. **E. Effect of ASMase deficiency and exogenous ceramide on TNFα mRNA production in RAW264.7 cells.** RAW264.7 cells were treated for 2 hours with LPS (10ng/ml) alone or in the presence of either desipramine (25μM), C_2-ceramide (60μM), or bacterial sphingomyelinase (0.1units/ml). The steady-state levels of TNFα mRNA were determined by RT-PCR.

CHAPTER FOUR

Role of ASMase and ceramide in the post-translational regulation of TNFα synthesis in response to LPS

A. Introduction

The concept of post-translational regulation of TNFα secretion in response to LPS has been proposed more than 20 years ago [112]. However, the mechanisms which regulate the rates of trafficking, processing, and release of TNFα (Fig. 4.1) remain mostly unknown.

In mice, TNFα mRNA is translated into a 26kD precursor most of which is associated with the Golgi complex [113]. Part of the newly synthesized protein is immediately N-glycosylated [77]. proTNFα residing in Golgi is selected as a cargo for the golgin p230-positive vesicles [114] which fuse with rab11 recycling endosomes *en route* to the plasma membrane [115]. The precursor is not delivered randomly to the cell surface but it is highly concentrated in the phagocytic cups [116] where it is integrated as a type II membrane protein. The proteolytic cleavage of proTNFα to the biologically active 17kD sTNFα is inhibited by cholesterol depletion and hydroxymate inhibitors, suggesting that it takes place in cholesterol-rich membrane domains and the enzyme responsible for proTNFα shedding is a metalloprotease [116-118]. Indeed, it was confirmed that at the membrane, proTNFα ectodomain is cleaved by TACE, which is a member of the ADAM (A Disintegrin And Metalloproteinase) family of proteases [119]. In addition to TNFα, TACE also processes the two TNFα receptors (p55 and p75), transforming growth factor alpha (TGFα), L-selectin, and other secretory proteins.

Recent studies on TNFα processing revealed that TACE activity might be a rate-limiting step in TNFα secretion [120]. For example, it was reported that a substantial portion of proTNFα that was not cleaved by TACE was rapidly

internalized and either degraded in the lysosomes or recycled back to the plasma membrane [121, 122].

TACE mRNA is expressed in most tissues and gives rise to an inactive zymogen. Removal of the autoinhibitory prodomain occurs in a late Golgi compartment, most likely by the action of furin or a related protease [123]. Only processed TACE appears on the cell surface [124]. The stimulation of cells with various agents can increase the rate of shedding. However, the mechanisms underlying the regulation of TACE activity remain poorly understood. A role of the cytoplasmic domain was suggested in response to intracellular signaling events such as MAP kinase activation [125]. In favor of this is the finding that the cytoplasmic domain is phosphorylated in response to shedding activators [126]. Nevertheless, TACE constructs lacking the entire cytoplasmic domain are fully functional in shedding [127], indicating that other mechanisms must be also involved. Another important question is what determines substrate selectivity of the enzyme. Since the sequences cleaved in various substrates are highly variable it was proposed that the distance between the membrane domain and the cleavage site is important. However, interactions distal to the cleavage site also appear to be required [127]. Finally, there is evidence to suggest that spatial segregation or proximity of enzyme and substrate within the plasma membrane may control some shedding activity.

At the plasma membrane, TACE activity appears to depend upon compartmentalization of the protein in the ordered lipid domains. It is controversial, however, whether localization in the ordered lipid rafts is linked to stimulation or to inhibition of activity. It is also not known whether these observed changes reflect differences in the co-localization of TACE and its substrates, or a conformational change and catalytic activation of the enzyme.

Lipid rafts are loosely defined as domains on the plasma membrane that are enriched in sphingomyelin and cholesterol. They constitute a unique liquid ordered environment that includes or excludes specific subsets of proteins and lipids. Sphingomyelin and cholesterol have a unique relationship in mammalian cells that involves physical and metabolic interactions. First and foremost, the presence of cis-, but not trans- double bond in the sphingoid moiety of

sphingomyelin makes the molecule “flat”. This allows for close hydrophobic interaction between sphingomyelin and cholesterol, evident by their ability to aggregate in domains that exclude other lipids. Furthermore, the importance of a hydrogen bond formed between the amide nitrogen of sphingomyelin and the oxygen atom in the cholesterol hydroxyl group has also been suggested as a structural basis for sphingomyelin:cholesterol complex formation [128, 129].

Translocation of ASMase to the plasma membrane leads to hydrolysis of sphingomyelin and generation of ceramide, followed by exclusion of cholesterol from the rafts and formation of large ceramide-enriched macrodomain platforms [130].

Interestingly, the two lipids, sphingomyelin and cholesterol, affect each other’s metabolism. Lowering of the content of sphingomyelin at the plasma membrane dramatically alters the cholesterol distribution by mobilizing plasma membrane cholesterol for esterification [43, 131], by decreasing sterol biosynthesis [43, 132], and by activating the synthesis of steroid hormones in steroidogenic cells [133]. The addition of cholesterol to cultured fibroblasts inhibits their ASMase activity. Conversely, the loss of ASMase activity leads to accumulation not only of sphingomyelin but also cholesterol [134], indicating that there is a regulatory mechanism which maintains a fixed ratio of sphingomyelin to cholesterol. Furthermore, the addition of bSMase that hydrolyses the SM at the plasma membrane, stimulates cholesterol translocation from the plasma membrane to the endoplasmic reticulum where it suppresses cholesterol synthesis by inhibiting HMG-CoA reductase [135].

Overall, it is fairly well established that sphingomyelin and cholesterol levels at the plasma membrane are coordinately regulated and affect signaling events by re-organizing the signaling domains, e.g. lipid rafts. This may have interesting implications for the regulation of TACE activity, since it has already been suggested that sequestration of TACE in the sphingomyelin:cholesterol rafts is rate-limiting for its activity [120].

Recent studies in mice have linked ASMase deficiency to increased susceptibility to viral and bacterial infections [55, 56]. ASMase-deficient macrophages exhibit a slow rate of elimination of pathogens like *L.*

monocytogenes, alphavirus Sindbis and *P. aeruginosa*, caused by a protracted phago-lysosomal fusion and membrane budding. A defect in the rab4 recycling pathway has been identified in $asm^{-/-}$ fibroblasts isolated from patients with Niemann-Pick Disease type A [136]. Together these studies suggest that in addition to a role in signaling, ASMase might be involved in the regulation of membrane fusion by regulating membrane fluidity and/or curvature.

B. Materials and Methods

Materials

Ammonium chloride was purchased from Sigma-Aldrich Co. (St. Louis, MO). Fluorogenic Peptide Substrate III and anti-TNFα used for immunofluorescence came from R&D Systems (Minneapolis, MN). TAPI-1 (N-(R)-[2-(Hydroxyaminocarbonyl) methyl]-4-methylpentanoyl-L-naphtyl-alanyl-L-alanine, 2-aminoethyl amide; TNFα Protease Inhibitor-1) was from Calbiochem (La Jolla, Ca). D-erythro-sphingosine was from Avanti Polar Lipids (Alabaster, AL). Anti-EEA1 and anti-TACE antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Litter-matched $asm^{+/+}$ and $asm^{-/-}$ mice were maintained as described earlier (Chapter three, Section B) and used for experiments.

SDS-PAGE and Western blotting

Please refer to Chapter Two, Section B for details in performing SDS-PAGE and Western blotting.

ELISA

The levels of TNFα in the medium from $asm^{+/+}$ and $asm^{-/-}$ miacrophages were determined by ELISA as described in Chapter one, Section B.

TACE activity assay

Macrophages were resuspended in 500μl detergent free 25mM Tris (pH 8.0) and homogenized by passing through a 26G needle several times. A total of 5-10μg protein was incubated with 10μM fluorogenic substrate III (Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2) in 25mM Tris, pH 8.0 (100μl final volume). The fluorescence was analyzed with excitation at 320nm and emission at 405nm.

Indirect Immunofluorescence

Cells were cultured on coverslips and treated as indicated. Fixation was performed for 5 min in cold methanol (permeabilized cells) or for 30 min in 3% formaldehyde (non-permeabilized cells). After blocking the non-specific binding with appropriate serum (2%), the proteins of interest were labeled with anti-TACE (1:50), anti-TNFα (1:50) or anti-EEA1 (1:100) polyclonal antibodies. The immune complexes were visualized with FITC-conjugated goat anti-rabbit IgG or TRITC-conjugated bovine anti-goat IgG. In the co-localization studies, the primary antibodies were added sequentially.

Statistical analysis

Please refer to Chapter Two, Section B.

C. Results

Kinetics of post-translational processing of TNFα in primary macrophages

To study the post-translational processing of TNFα, the conversion of proTNFα to sTNFα was monitored by Western blotting in primary macrophages and RAW264.7 cells. As anticipated, neither proTNFα nor sTNFα forms were detected in non-stimulated peritoneal macrophages from both genotypes (Fig.4.2 A, and B). Substantial accumulation of sTNFα in the medium of LPS-treated macrophages was noticeable at 6 hours after stimulation and continued for at least 20 hours. The amounts of sTNFα were higher in $asm^{-/-}$ than in $asm^{+/+}$ cells, which is in excellent agreement with the differences seen by ELISA. Surprisingly, we were not able to detect any proTNFα in the cellular lysates (Fig. 4.2A), which is indicative that the steady-state levels of proTNFα were very low. However, co-treatment with ammonium chloride, which blocks the activity of many lysosomal hydrolyses led to very substantial accumulation of the precursor protein inside the cells (Fig. 4.2 D), while having no effect on the levels of sTNFα in the medium (Fig. 4.2 E). These results are in good agreement with earlier observations by other groups [122], indicating that proTNFα that is not processed by TACE is internalized and degraded in the lysosomes. Our study further suggests that at least half of the newly produced proTNFα undergoes such degradation, based on calculations of the volumes that were loaded. Together, these observations support the conclusion that in peritoneal macrophages the activity of TACE is the rate-limiting factor in TNFα processing and that at least in primary macrophages, the lysosomal proteolysis of non-processed TNFα is very substantial and rapid.

Similar studies were also conducted in the macrophage cell line, RAW264.7. As discussed in chapter 2, LPS treatment induced TNFα production, which was readily detected in the medium as well as in the cells. In contrast to peritoneal macrophages the levels of proTNFα in the cells were substantial. Importantly, inhibition of ASMase activity by desipramine had only a slight effect on proTNFα and sTNFα (Fig. 4.3 A, B, C). Furthermore, unlike primary macrophages, the addition of ammonium chloride to RAW 264.7 cells to block lysosomal proteolysis of TNFα, did not influence the levels of proTNFα detected in cell lysates (Fig. 4.3 E). Together, these results indicate that there is a

significant difference between primary peritoneal macrophages and RAW264. 7 cells in the post-translational regulation of TNFα production. Apparently, in RAW264.7 cells proTNFα did not undergo efficient lysosomal proteolysis and its levels were not affected by inhibition of lysosomal proteases. Because of these differences between primary cells and the cell line, the latter was not used any further.

Asm$^{-/-}$ macrophages have higher activity of TACE, which is inhibited by an increase in ceramide content of the cells

To test directly whether increased TNFα production in asm$^{-/-}$ was caused by higher activity of TACE, the latter was measured using fluorogenic substrate containing the TNFα cleavage site. After identifying the range of linearity for the *in vitro* assay (Fig. 4.4A, and B), the TACE activity was measured in detergent-free lysates from asm$^{+/+}$ and asm$^{-/-}$ cells. Both, LPS- and control-treated macrophages isolated from asm$^{-/-}$ mice exhibited 2 to 3 fold higher activity as compared to their respective counterparts isolated from the asm$^{+/+}$ mice. These differences suggest that ASMase-derived ceramide might be a negative regulator of TACE (Fig. 4.4C, and D).

To test this, the levels of cellular ceramide were elevated by the addition of bacterial sphingomyelinase or cell-permeable ceramide analogue, C_2-ceramide, to the cultured cells. Both treatments inhibited TACE activity but ceramide-mediated inhibition was more than 80% (Fig. 4.4E, and F). To test whether ceramide has a direct effect on TACE activity, it was added directly to the assay mixture. No inhibitory effect, however, was observed (Fig. 4.5.A). One possible explanation for the differences in ceramide capacity to inhibit TACE in cells but not *in vitro* was that in cells ceramide was metabolized. To test this, two ceramide metabolites, sphingosine and dihydroceramide, were used. However, these treatments also failed to inhibit TACE (Fig. 4.5. B, C). Together these results suggest that ceramide has no direct effect on TACE, but may influence its activity by reorganizing the membrane bilayer and/or its intracellular localization.

Such sensitivity of TACE to the lipid environment was also indicated by the inhibitory effect that the addition of Triton X-100 to the TACE assay mixture had on the measured activity (Fig. 4.5 D). To that extent, we also observed differences in TACE sub-cellular localization between the two genotypes. While in $asm^{+/+}$ macrophages TACE was localized in a Golgi-like compartment in close proximity to the nucleus, in $asm^{-/-}$ cells a uniform and diffused staining was observed throughout the cell body (Fig. 4.6 B). Studies using quantitative real-time PCR revealed that $asm^{-/-}$ cells do not express higher levels of TACE mRNA (Fig. 4.6 A), thus excluding the possibility that the genotype-specific difference in activity and localization reflected differences in protein expression.

Surprisingly, TACE activity was not sensitive to stimulation with LPS. However, this could be a consequence of the specific assay conditions because the endogenous substrate, which is absent in the control-treated cells, but abundant in the LPS-treated cells may be an efficient competitor for the exogenous substrate during an *in vitro* assay.

Effects of ASMase deficiency on the sub-cellular distribution of TNFα

Apparently, ASMase deficiency affected step(s) in the processing of proTNFα to the sTNFα form. To directly monitor proTNFα intracellular trafficking to and from the plasma membrane, indirect immunofluorescence was employed. This approach allowed the detection of substantial production of TNFα inside the cells, mostly in ER/Golgi compartment (in permeabilized cells), and an accumulation at the plasma membrane (in non-permeabilized cells) in response to stimulation with LPS (Fig. 4.7A, and B). However, several apparent differences were noticeable in $asm^{-/-}$ macrophages: (i) Seemingly, more TNFα was present throughout the cytosol, and (ii) TNFα-positive vesicular structures were detected in a close proximity to the plasma membrane. Co-treatment with TAPI-1, a TACE inhibitor, increased the number of these TNFα positive vesicles in $asm^{-/-}$ but not in $asm^{+/+}$ cells (Fig. 4.7 A and Fig. 4.8 B). Therefore, these vesicular structures in $asm^{-/-}$ cells most likely contained TNFα designated for TACE-mediated cleavage.

Having in mind that TACE activity was higher in the $asm^{-/-}$ macrophages, the fact that TAPI-1 sensitive pool of TNFα was present in $asm^{-/-}$ but not in $asm^{+/+}$ cells may indicate that more proTNFα molecules were recycled back to the plasma membrane and underwent TACE-mediated processing in the $asm^{-/-}$ cells than in $asm^{+/+}$ cells. To test this, cells were stained for EEA1, a marker for early endosomes. Notably, $asm^{-/-}$ macrophages had a higher number of early endosomes as compared to $asm^{+/+}$ macrophages (Fig. 4.7 C, and Fig. 4.8 C), indicative of more active recycling pathways. To test whether the endosomes are indeed as a reservoir of proTNFα for plasma membrane recycling, co-localization studies were done. More TNFα-positive vesicles co-localized with EEA1 in LPS treated $asm^{-/-}$ cells as compared to $asm^{+/+}$ cells (Fig. 4.8A, and B). The number of these double-positive vesicles increased in the presence of TAPI-1 only in $asm^{-/-}$ cells, but not in $asm^{+/+}$ cells (Fig. 4.8 D). Treatment with ammonium chloride led to a significant increase in both TNFα-positive and double-positive cellular compartments (Fig. 4.8 D). Importantly, the effects of ammonium chloride were more pronounced in the $asm^{+/+}$ than in $asm^{-/-}$ cells. Together these results consistently show that the early endosomes contained proTNFα that is salvaged from lysosomal proteolysis and is destined for recycling and TACE-mediated processing. ASMase activity plays an important role in partitioning proTNFα between these two pathways.

Effects of exogenous sphingomyelinase on TNFα processing

To test whether ASMase and ceramide are directly involved in regulating TNFα recycling, rescue experiments were done. $Asm^{-/-}$ cells were treated with exogenous sphingomyelinase to increase ceramide at the plasma membrane. This was sufficient to bring the sTNFα level close to those measured in $asm^{+/+}$ cells (Fig. 4.9A, B). Furthermore, sphingomyelinase treatment also significantly decreased the number of early endosomes detected in $asm^{-/-}$ cells (Fig. 4.9 C). These results not only link SMase and ceramide to early endosomes biogenesis

but also reveal a correlation between the number of early endosomes and the levels of sTNFα released in response to LPS.

Genotype-specific differences in cytokine release *in vivo*

To determine the effect of ASMase deletion on TNFα production *in vivo*, litter-matched $asm^{+/+}$ and $asm^{-/-}$ mice were injected with saline or LPS (5.8mg/kg of body weight). TNFα and IL-1β were undetectable in saline-injected mice of both genotypes. The administration of LPS led to a sharp increase in serum levels of both cytokines. However, in ASMase-deficient mice, serum TNFα reached concentrations that were 10 to 15 fold higher than those measured in sera from $asm^{+/+}$ mice (Fig. 4.10 A), while the levels of IL-1β (Fig. 4.10 B) and IL-6 (data not shown) were similar for both genotypes. These data are in agreement with the genotype-based differences in TNFα secretion observed in isolated peritoneal macrophages and indicate that ASMase deficiency has a specific effect on the regulation of TNFα *in vivo*.

D. Discussion

The results presented in this chapter provide evidence that ASMase and ceramide regulate the rate of post-translational processing and secretion of TNFα after LPS stimulation.

Two distinct mechanisms seem to be involved. First, ASMase-derived ceramide apparently functions as a negative regulator of TACE activity. This is supported by the inhibition of TACE activity observed after the treatment of macrophages with exogenous ceramide or bacterial SMase, both of which increase ceramide content of the plasma membrane. Consistently, the intrinsic activity of TACE was higher in $asm^{-/-}$ macrophages as compared to $asm^{+/+}$ cells. Most likely, ceramide and ASMase modulate TACE activity by changing the membrane microenvironement of the enzyme, because ceramide was effective only when added to cultured cells but not directly to the assay mixture. As

discussed earlier, TACE is a transmembrane protein and, based on the complete loss of activity observed in the presence of detergent, seems to require an intact lipid environment for optimal activity. Notably, Tellier et al. found that the mature form of TACE, but not the inactive pro-form, is localized in the sphingomyelin:cholesterol-rich domains and co-purifies with caveolin 1 and flotilin-1. These authors further suggest that sequestration of TACE in the sphingomyelin:cholesterol rafts is rate-limiting for its activity [120]. This is in excellent agreement with our conclusion that ASMase and ceramide control a rate-limiting step in TNFα processing. It is of particular interest that treatment with SMase that not only hydrolyses SM, thereby depleting the plasma membrane of cholesterol, but also has an inhibitory effect of the endogenous TACE. It seems likely that the elevated sphingomyelin (and cholesterol) in $asm^{-/-}$ cells create a more "favorable" environment for TACE and, consequently, higher activity. Second, a seemingly independent mode of regulation was revealed by our indirect immunofluorescent studies. These experiments suggest that ASMase also has an affect on the fate of intracellular proTNFα in peritoneal macrophages. It has been proposed previously that proTNFα that is not immediately cleaved by TACE is internalized and either degraded in the lysosomes or recycled back to the plasma membrane, where it can be utilized for TACE-mediated cleavage. Our results strongly support such a scenario and provide initial evidence that the partitioning of the non-processed TNFα between these two pathways is modulated and may have a profound effect on the levels of TNFα secreted in a response to particular stimulus.

Consistent with previous studies implicating ASMase in the dynamics of vesicle fusion and fission during endocytosis, we also found that $asm^{-/-}$ macrophages have increased the number of early endosomes consistent with a defect in the endosome maturation to lysosomes. Furthermore, the increased co-localization of EEA1 and TNFα provides evidence that in the absence of ASMase, TNFα is sequestered in the early endosomes and likely recycled back to the plasma membrane and cleaved by TACE. Evidence for this comes from the observation that the inhibition of TACE by TAPI-1 caused significant elevation

in intracellular TNFα as well as TNFα/EEA1 co-localization only in the $asm^{-/-}$ but not in $asm^{+/+}$ cells. In contrast, in $asm^{+/+}$ cells similar increases were observed in the presence of ammonium chloride, indicating that a portion of intracellular TNFα, localized in the early endosomes undergoes TACE-mediated cleavage in $asm^{-/-}$ cells, but lysosomal clearance in $asm^{+/+}$ cells.

Membrane lipids and proteins are moved from the early endosomes back to the cell surface via two main routes: the rab4-dependent fast recycling pathway, and the rab11-mediated slow recycling pathway. Fibroblasts from Niemann-Pick Disease Type A patients seem to lack the rab4-mediated recycling, but exhibit a more active rab11-dependent pathway [136]. Therefore, the latter could be responsible for the increased TNFα recycling in $asm^{-/-}$ cells.

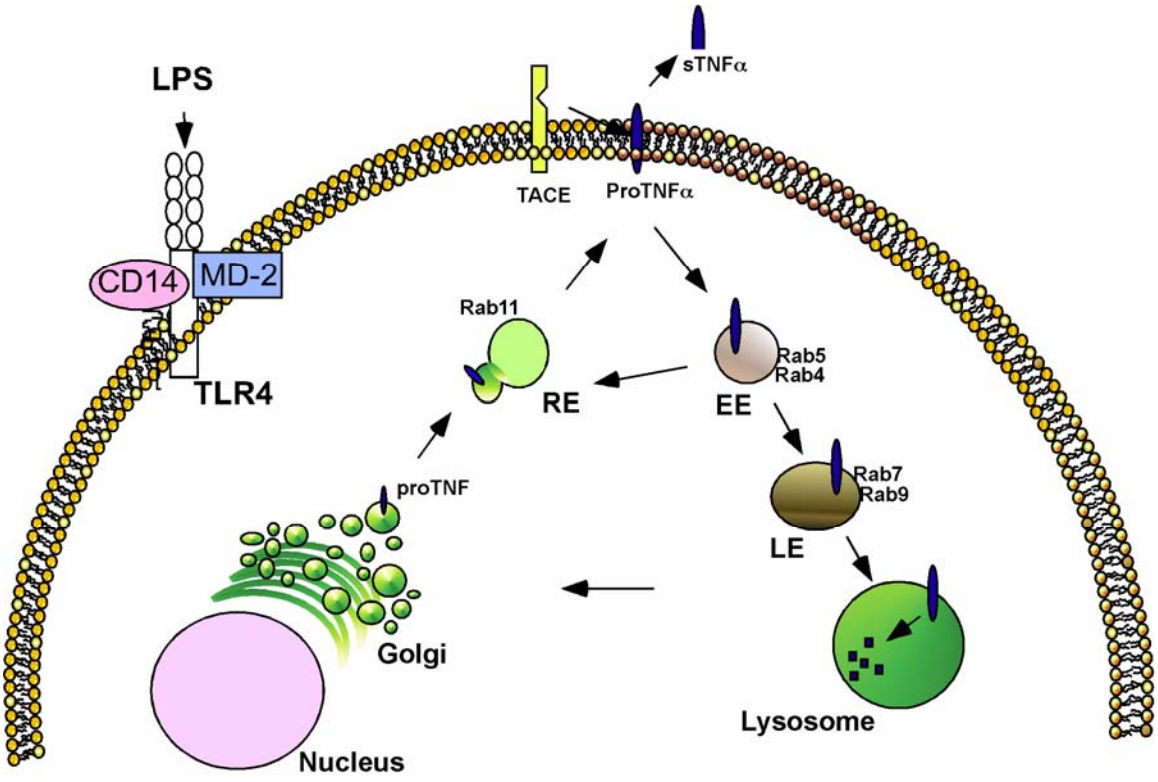


Figure 4.1. Post-translational processing of TNFα. TNFα is initially synthesized as 26kD precursor molecule (proTNFα). It is selected as a specific cargo of trans-Golgi vesicles, which fuse with rab11 recycling endosomes *en route* to the plasma membrane. At the plasma membrane proTNFα is cleaved by TACE at its ectodomain to release the soluble 17kD mature form (sTNFα). The uncleaved proTNFα is internalized and either degraded in the lysosomes or recycled back to the plasma membrane. *Abbreviations:* TLR4, Toll-like receptor 4; RE, recycling endosomes; EE, early endosomes; LE, late endosomes.

Primary peritoneal macrophages

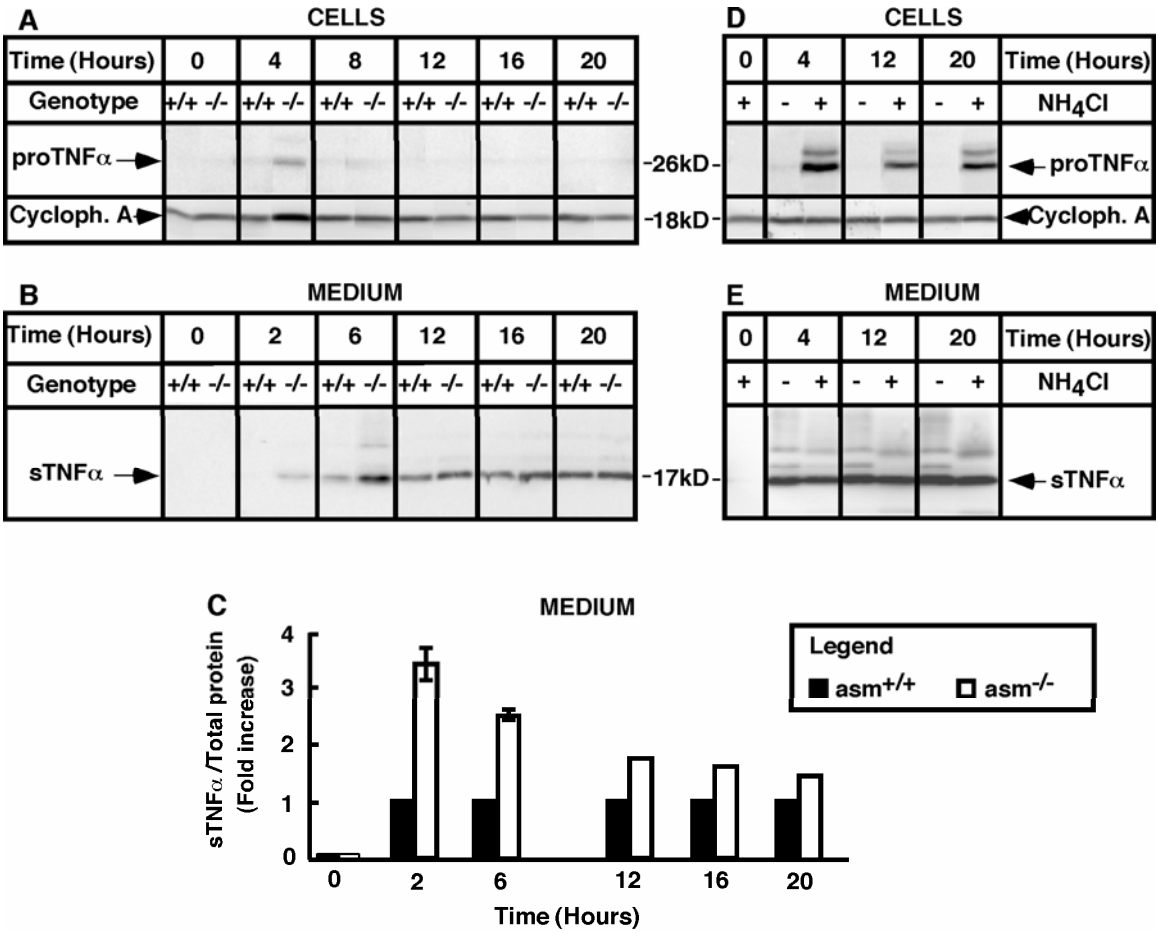


Figure 4.2. Post-translational processing of TNFα in primary peritoneal macrophages. Peritoneal macrophages were isolated from $asm^{+/+}$ and $asm^{-/-}$ mice and treated with LPS (10ng/ml) for the indicated times in the presence or absence of ammonium chloride (10mM). TNFα levels were determined in cell extracts (A, D) or medium (B, C, and E) by Western Blotting. Cyclophilin A levels were used to control for uniform loading. For quantification purposes, the data were shown as a ratio of the levels in the medium collected from $asm^{-/-}$ and $asm^{+/+}$ cells and are mean ± s.d. of three independent experiments.

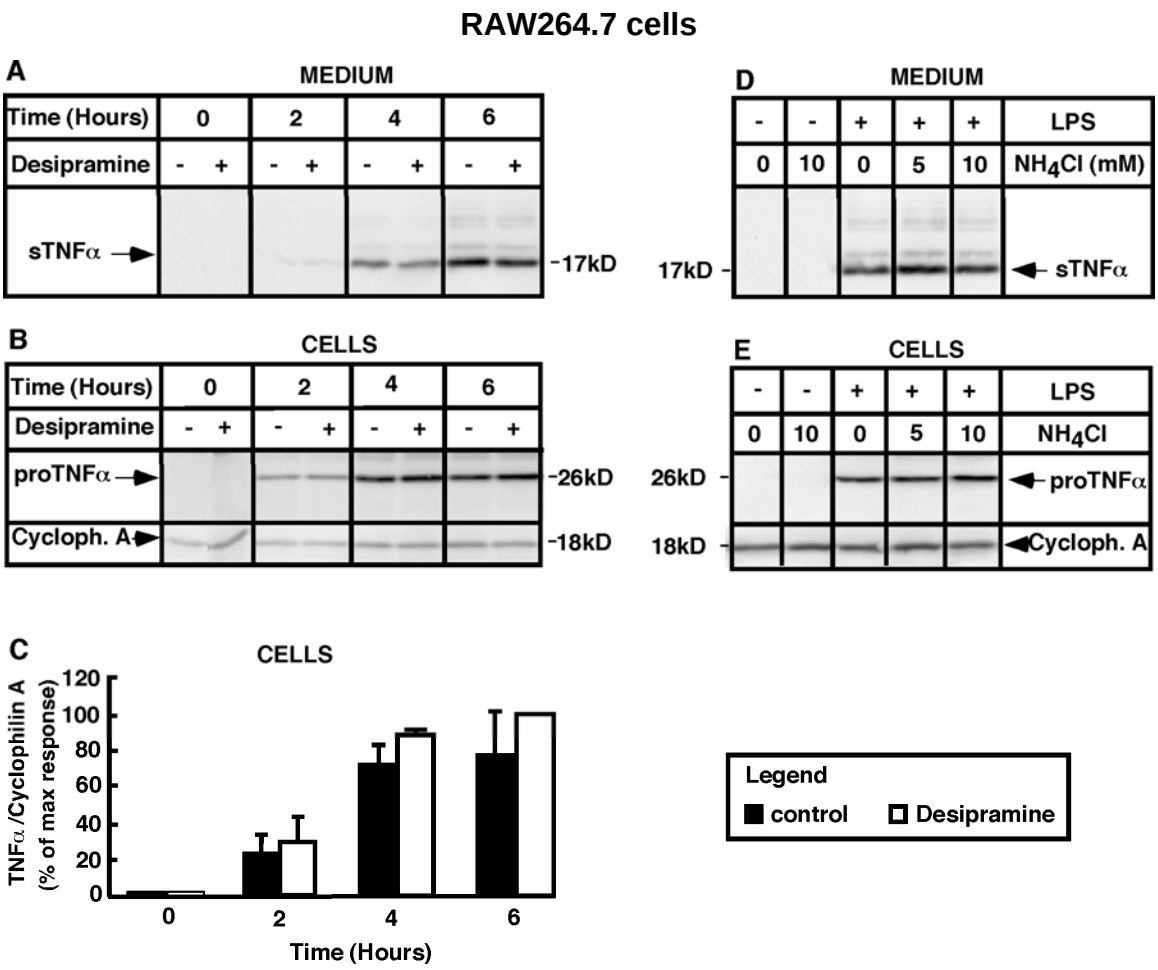


Figure 4.3. Post-translational processing of TNFα in RAW264.7 macrophage cell line. RAW264.7 cells were stimulated with LPS (10ng/ml) for the indicated time in the presence or absence of ammonium chloride (10mM). Where indicated pre-treatment with desipramine (25μM) was used to inhibit ASMase activity. TNFα levels were determined in cell extracts (B, C, E) or medium (A, and D) by Western Blotting. Cyclophilin A levels were used to control for uniform loading. For quantification purposes (C), the data were shown as a percent of the maximum response and are mean ± s.d. of two independent experiments.

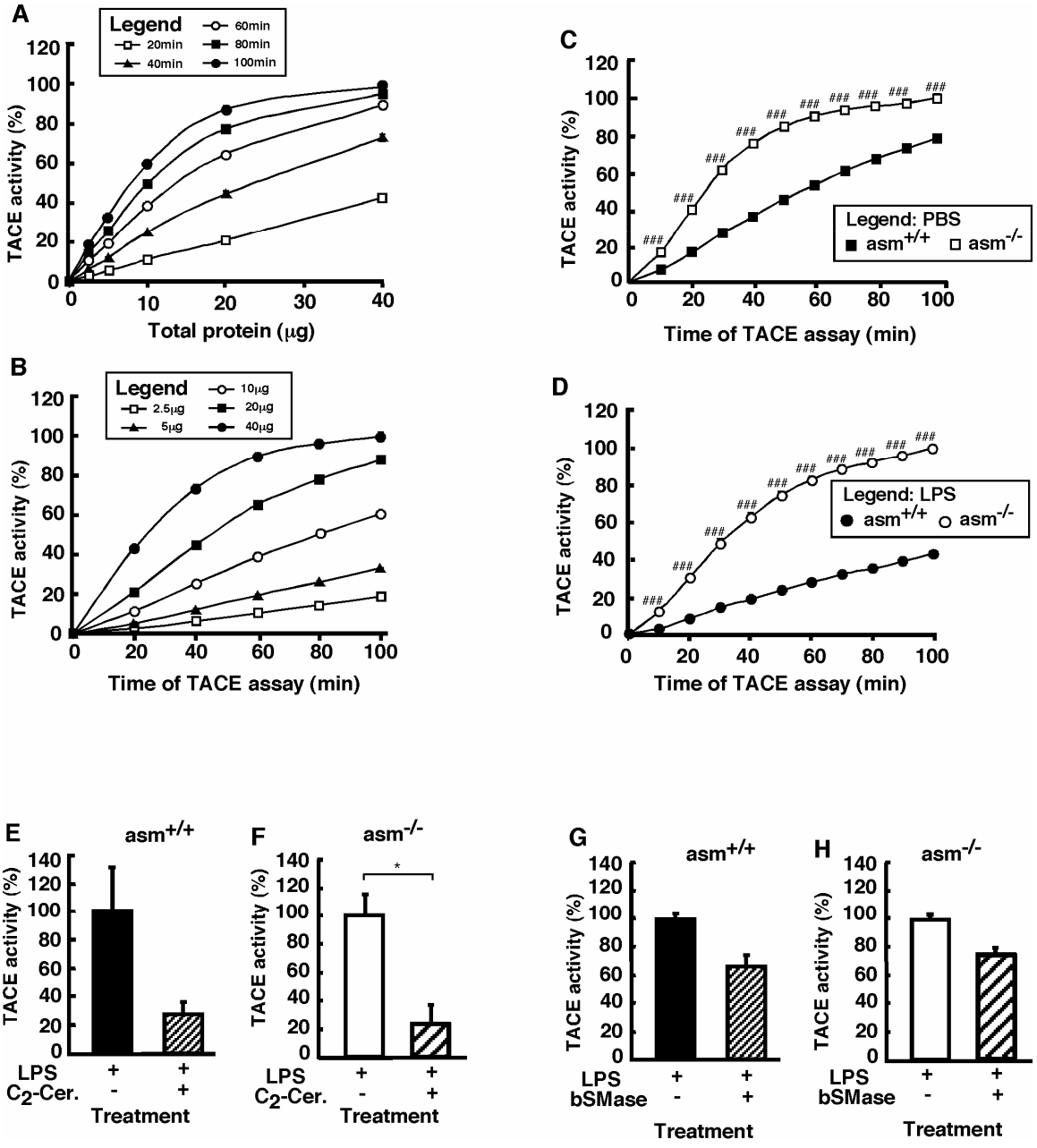


Figure 4.4. TACE activity in asm$^{+/+}$ and asm$^{-/-}$ macrophages. A, B. Test for linearity of TACE activity assay with protein and time. TACE activity measured *in vitro* in detergent-free lysates prepared from RAW264.7 cells. **C, D. Effect of ASMase deficiency on activity of TACE**. TACE activity is measured in lysates from asm$^{+/+}$ and asm$^{-/-}$ macrophages *in vitro*. Cells were treated with saline (C) or LPS (10 ng/ml, D) for 4 hours. Activity is shown as a percent of the

maximum value measured in each set of experiments, which were 3907 RFU and 3501 RFU for saline- and LPS-treated $asm^{-/-}$ macrophages, respectively. The significance of the interaction effect is shown (###, $p<0.001$) based on two-way ANOVA. **E-H**. **Effect of sphingomyelinase and ceramide on TACE activity.** $asm^{+/+}$ (E, G) and $asm^{-/-}$ (F, H) macrophages were treated with LPS (10ng/ml) for 4 hours in the presence of C_2-ceramide (60μM) (E, F), vehicle control (0.1% ethanol) or bacterial sphingomyelinase (0.1units/ml) (G, H). TACE activity is shown as percent of the value measured in vehicle-treated cells. The data are mean ± s.d. (n=3**)** and statistical significance of the treatment is shown (*, $p<0.05$) based on student t-test.

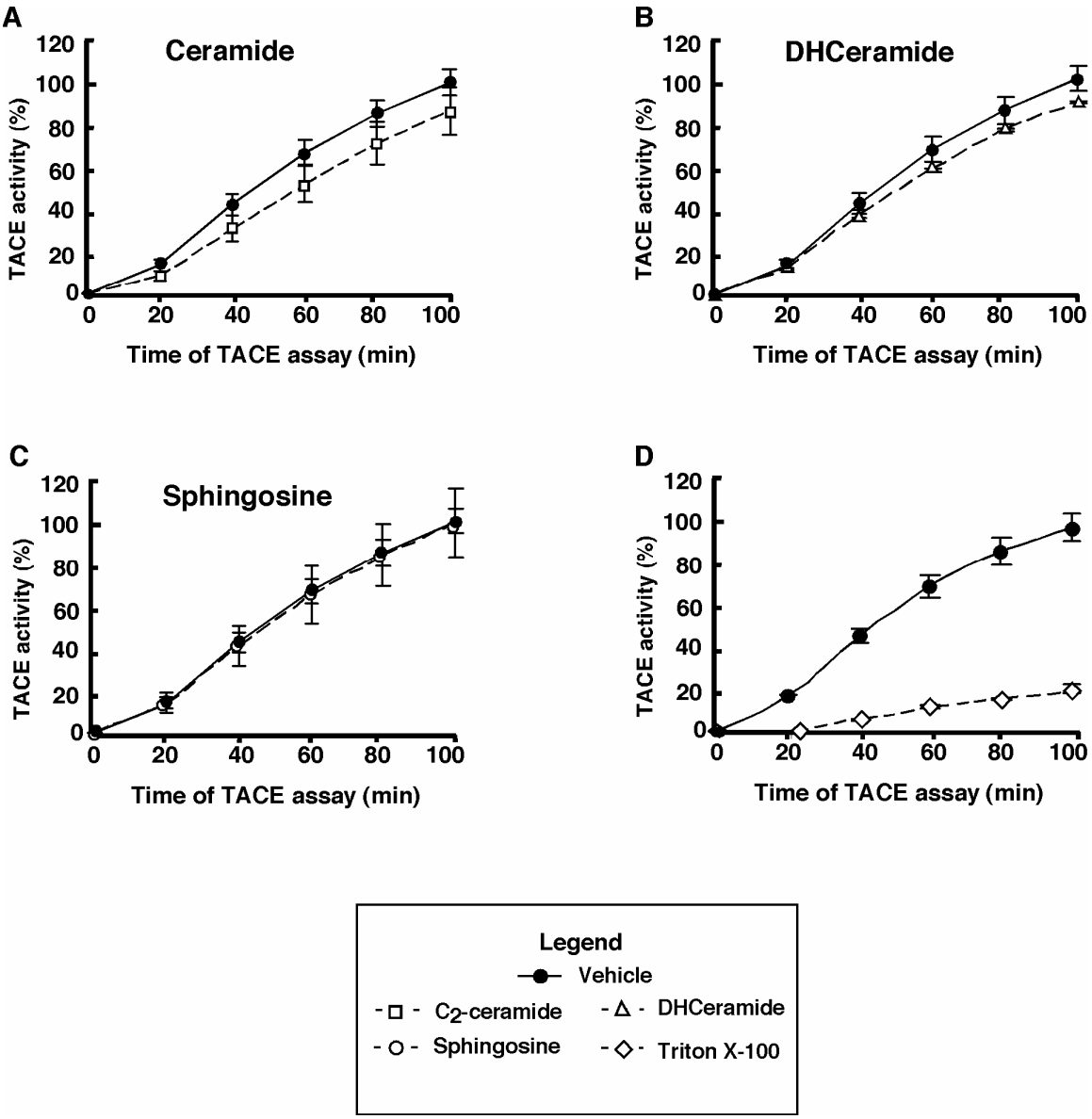


Figure 4.5. Effect of sphingolipids and detergent on TACE activity *in vitro.* **A-C Effect of Sphingolipids.** TACE activity was measured in detergent-free lysates from $asm^{+/+}$ macrophages in the presence of C_2-ceramide (A), Dihydroceramide (B), and Sphingosine (C) added directly to the reaction mixture of TACE activity assay. **D. Effect of Detergent**. TACE activity was measured in lysates from $asm^{+/+}$ macrophages in the presence (diamond) or absence (circle) of Triton X-100. TACE activity is shown as percent of the value measured in vehicle-treated cells.

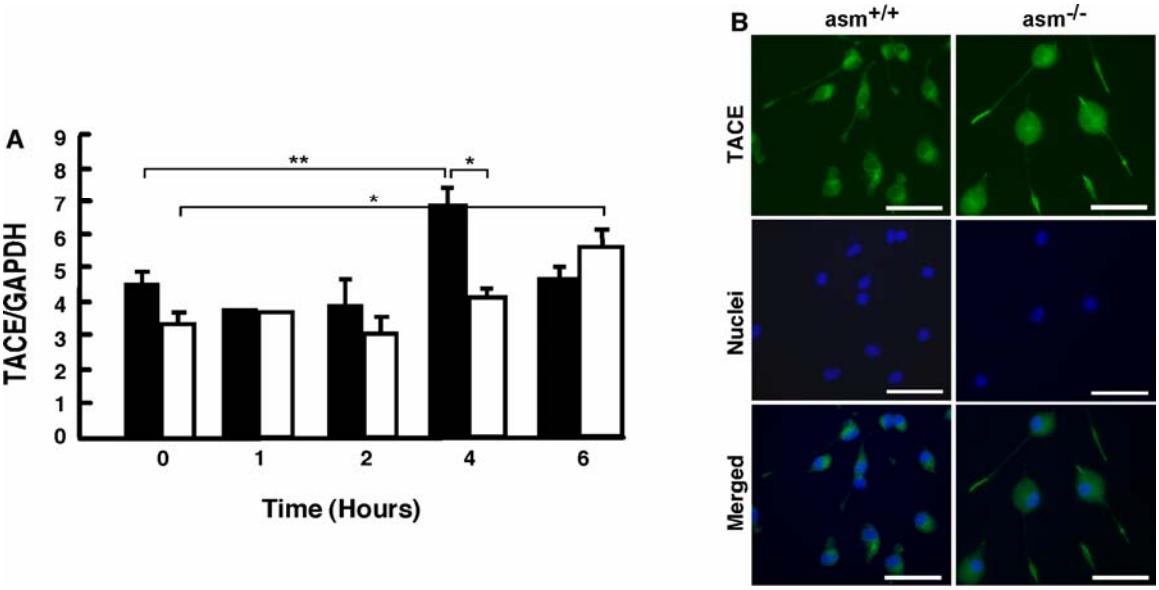
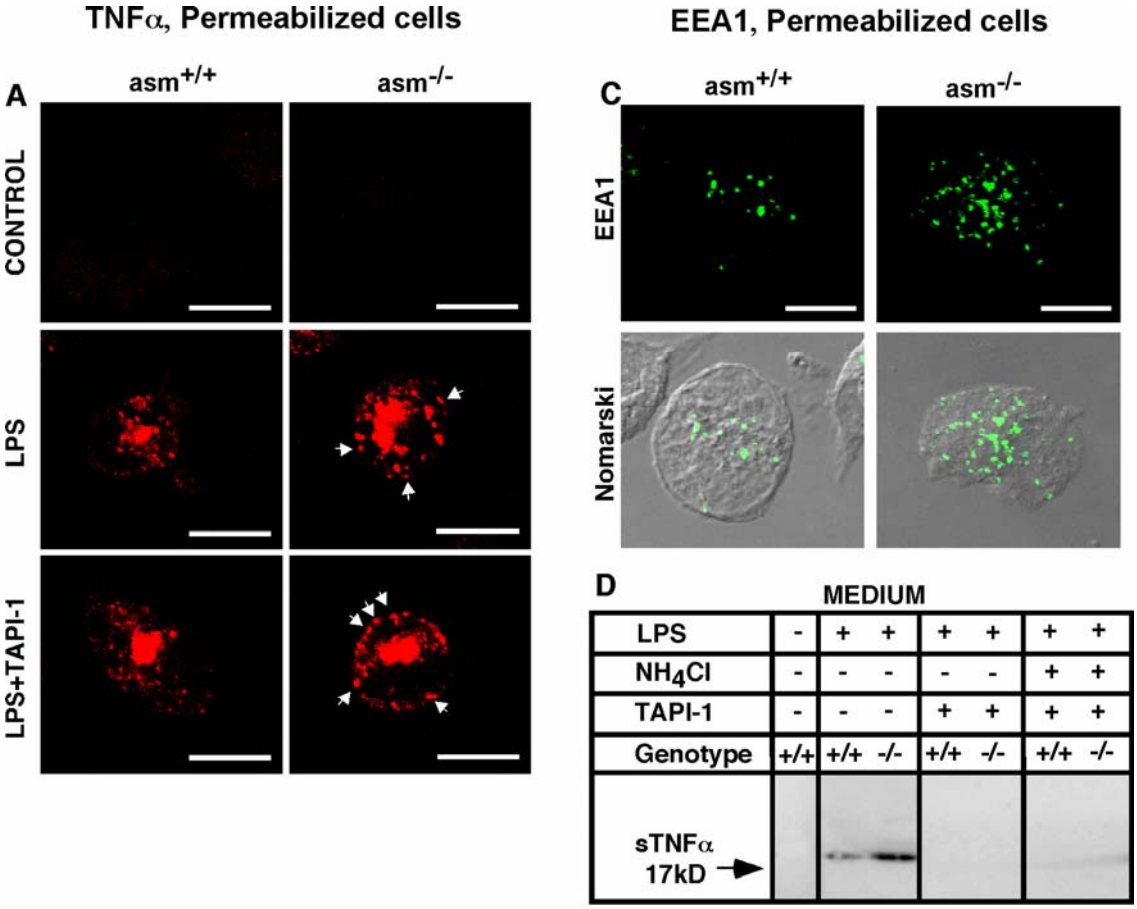


Figure 4.6. Expression and sub-cellular localization of TACE. **A. TACE mRNA levels.** Macrophages from $asm^{+/+}$ and $asm^{-/-}$ mice were isolated and treated with LPS (10ng/ml) for the indicated time. TACE mRNA levels were determined by real-time PCR and normalized to GAPDH mRNA in the same sample. The Data are presented as mean ± s.e.m. of three independent experiments. Statistical significance of the treatment effect is shown (**, $P<0.01$; *. $P<0.05$) based on two-way ANOVA. **B. Visualization of TACE.** The localization of TACE in permeabilized $asm^{+/+}$ and $asm^{-/-}$ was visualized using antibodies against mouse TACE and fluorescent microscopy. Representative image of three independent experiments is shown. Hoechst 33258 was used for staining the nuclei. Scale bar represents 20μm.


TNFα, Permeabilized cells
A
asm+/+
asm-/-
CONTROL
LPS
LPS+TAPI-1
EEA1, Permeabilized cells
C
asm+/+
asm-/-
EEA1
Nomarski
D
MEDIUM
LPS - + + + + + +
NH4Cl - - - - - + +
TAPI-1 - - - + + + +
Genotype +/+ +/+ -/- +/+ -/- +/+ -/-
sTNFα 17kD

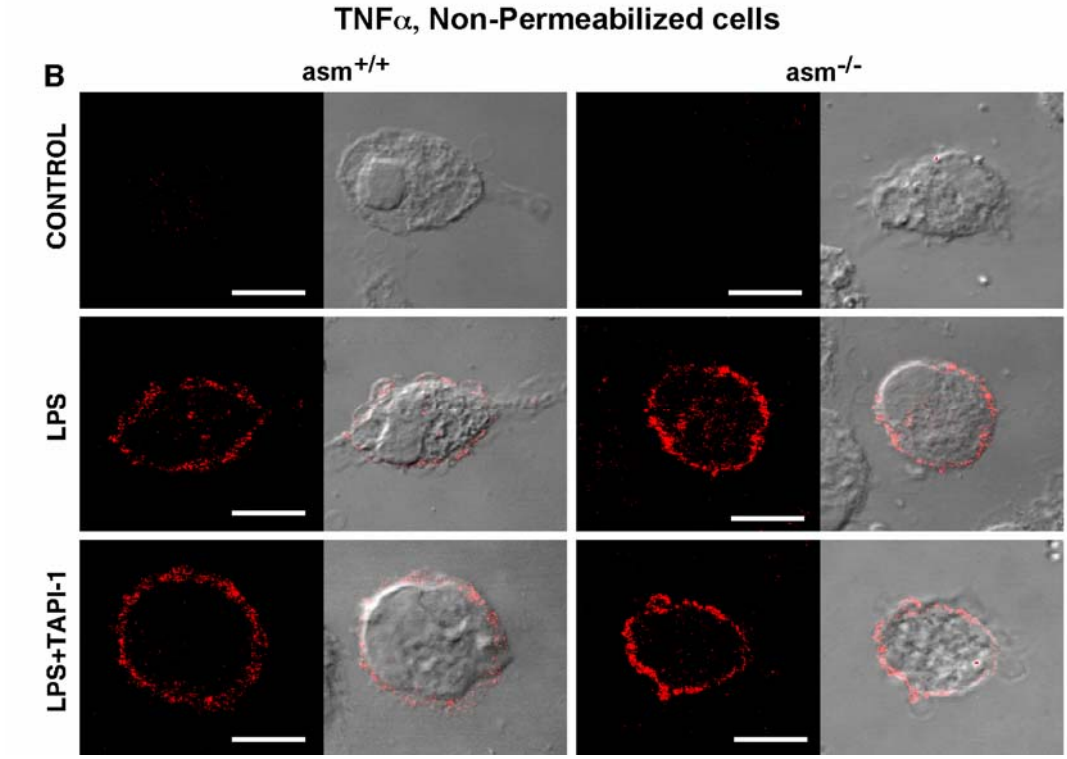

TNFα, Non-Permeabilized cells
B
asm+/+
asm-/-
CONTROL
LPS
LPS+TAPI-1

Figure 4.7. Sub-cellular localization of TNFα in $asm^{+/+}$ and $asm^{-/-}$ macrophages. Macrophages from $asm^{+/+}$ and $asm^{-/-}$ mice were isolated and treated with LPS (10ng/ml) in the presence and absence of TAPI-1 (20μM) and NH_4Cl (10mM) for 4 hours. TNFα was visualized in permeabilized (A) and non-permeabilized (B) cells using antibodies against TNFα and confocal microscopy. The arrows indicate TNFα-positive vesicles proximal to the plasma membrane that are visible in $asm^{-/-}$ cells. Antibodies against the endosomal marker EEA1 were used to visualize early endosomes (C). Western blot analyses of the cell culture medium that demonstrate the efficiency of the inhibitors (D). Transmitted light images (B, C) show cells morphology. The scale bar represents 10μm.

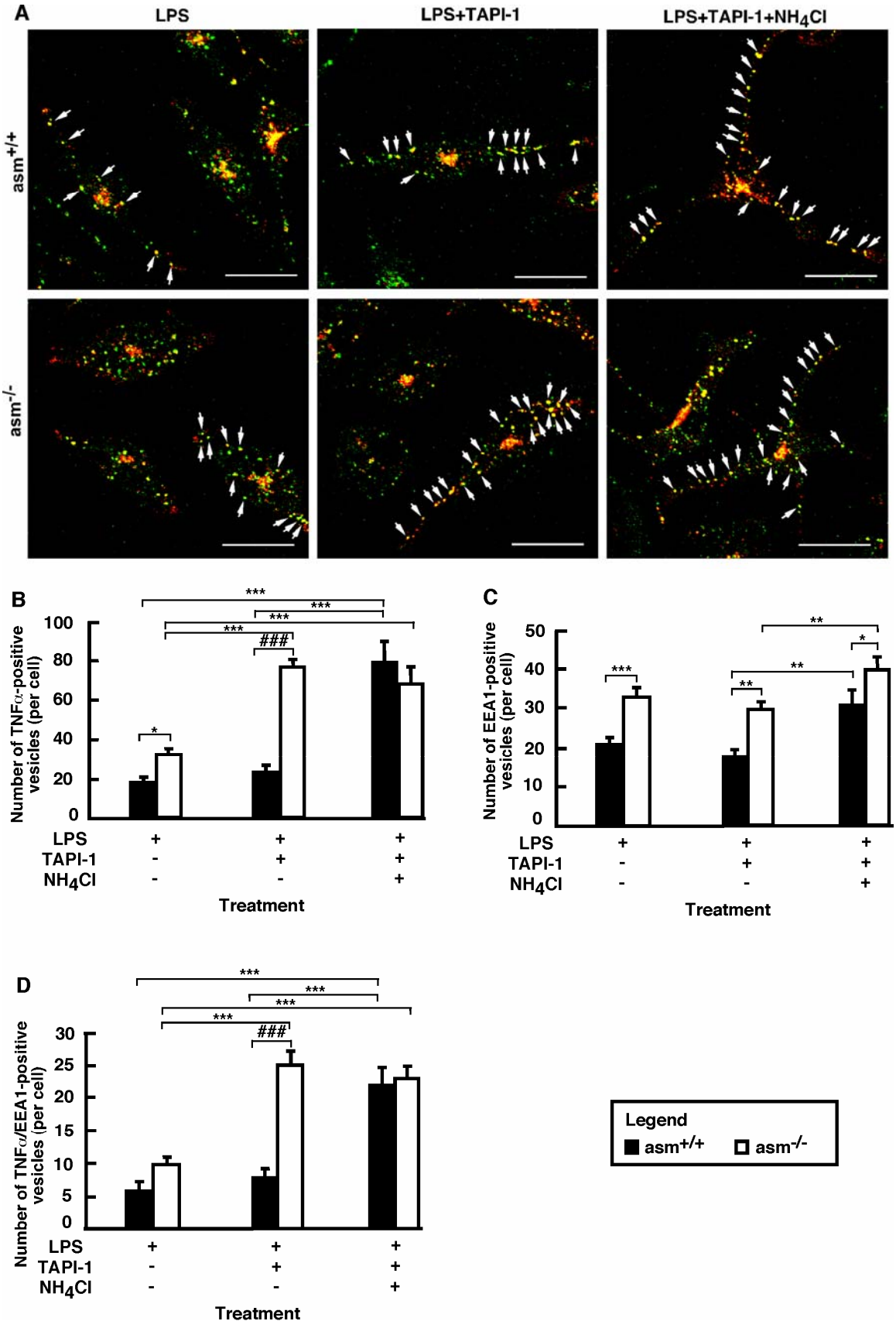

A
LPS
LPS+TAPI-1
LPS+TAPI-1+NH4Cl
asm+/+
asm-/-
B
Number of TNFα-positive vesicles (per cell)
LPS
TAPI-1
NH4Cl
Treatment
C
Number of EEA1-positive vesicles (per cell)
LPS
TAPI-1
NH4Cl
Treatment
D
Number of TNFα/EEA1-positive vesicles (per cell)
LPS
TAPI-1
NH4Cl
Treatment
Legend
asm+/+
asm-/-

Figure 4.8. Co-localization of TNFα and EEA1 in $asm^{+/+}$ and $asm^{-/-}$ macrophages. Peritoneal $asm^{+/+}$ and $asm^{-/-}$ macrophages were treated with LPS (10ng/ml) for 4 hours. Permeabilized cells were probed sequentially for TNFα (red) and EEA1 (green). Images were collected using confocal microscopy and merged (yellow). Only merged images are shown and the TNFα/EEA1 co-localization in a single cell for each panel is depicted by arrows. For quantification purposes, the bulk fluorescence visible in the center of each cell was excluded and the number of TNFα (B)-, EEA1(C)- and double (D)-positive vesicles (size bigger than 4 pixels) was counted in 10 cells of each slide. Data shown are average ± s.e.m. Statistical significance of the main effect (***<0.001; **<0.01; *<0.05) and interaction effect (### < 0.001) was determined by two-way ANOVA. The scale bar represents 20μm.

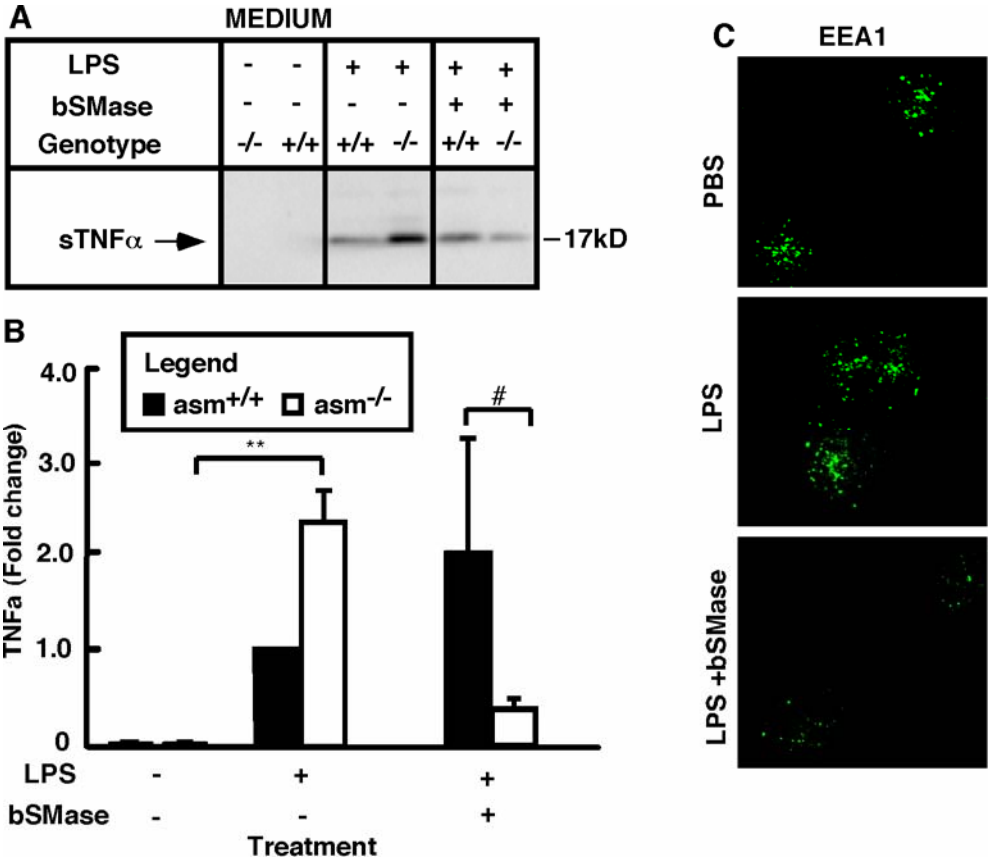


Figure 4.9. Effect of exogenous sphingomyelinase on sTNFα and EEA1 in asm$^{-/-}$ macrophages. Elicited peritoneal macrophages from asm$^{+/+}$ and asm$^{-/-}$ mice were treated for 4 hours with 10ng/ml LPS alone or in combination with 0.1units/ml bacterial sphingomyelinase. **A. Levels of TNFα in medium** based on Western blotting. **B. Quantification of sTNFα in the medium**. The graph represents the mean values ± s.d. of intensity of TNFα bands from three independent experiments. **C. Visualization of the early endosomes.** Immunofluorescent images depicting EEA1-positive early endosomes (green) in permeabilized macrophages. Statistical significance of the main effect (**, $P<0.01$) and the interaction effect of the treatment and the genotype (#, $P<0.05$) are shown based on two-way ANOVA.

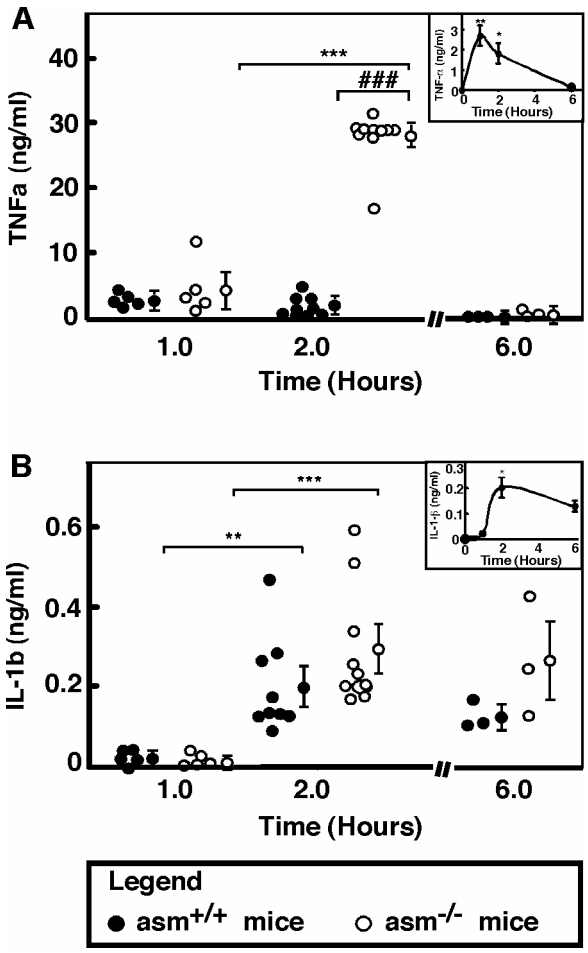


Figure 4.10. Cytokine levels in LPS-injected asm$^{+/+}$ and asm$^{-/-}$ mice.

Mice were injected i.p. with LPS (5.8mg/kg body weight) or saline. Serum was collected at the indicated times and the levels of TNFα (A) and IL-1β (B) were measured by ELISA. Data for LPS-injected asm$^{+/+}$ (filled circles) and asm$^{-/-}$ (open circles) are shown. The levels of the cytokines in saline-injected animals were below the detection levels. Data for individual animals are shown and are the average of identical measurements done in triplicates. The group average $\pm$ s.e.m. is shown on the side. The significance of the main effects of genotype and LPS (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$) and the interaction effect (###, $P<0.001$) is based on two-way ANOVA with Bonferroni post-test analysis. The inserts are representation of the data from asm$^{+/+}$ mice on a smaller scale.

CHAPTER FIVE

Conclusion

A. Summary of findings

This study provides evidence that ASMase and ceramide act as negative regulators of LPS-induced TNFα secretion both *in vivo* and *in vitro*.

Studies in isolated peritoneal macrophages showed that in ASMase deficient cells LPS induced up to 3 fold higher levels of sTNFα compared to the control cells. Exogenous ceramide was sufficient to suppress TNFα secretion confirming the specificity of this effect.

Further mechanistic investigations in peritoneal macrophages as well as in RAW264.7 macrophage cell line revealed that ASMase deficiency does not affect the rate of IRAK-1 degradation and the activation pattern of ERK and JNK. Moreover, the steady-state levels of TNFα mRNA were not influenced by ceramide or the lack of ASMase activity. Altogether, these results indicate that ASMase affects step(s) of TNFα production downstream of mRNA synthesis.

Detailed studies on the post-translational regulation of TNFα secretion further revealed that in macrophages post-translational processing is a major determinant of the amount of sTNFα secreted in response to LPS. Importantly, for the first time it was demonstrated that a substantial amount of proTNFα undergoes proteolysis in an acidic compartment, most likely lysosomes, since treatment with the lysosomotropic agent ammonium chloride caused a dramatic acumulatin of proTNFα in the cells. These results further suggest that the activity of TACE is rate-limiting for the production of sTNFα and is likely under tight regulation.

Indeed, ASMase and ceramide seem to affect the cleavage of proTNFα to the bioactive mature form, sTNFα, by acting as negative regulators of TACE activity. Similarly to the effect on TNFα secretion, ASMase deficiency was linked to 3 fold increase in TACE activity and this effect was abolished after treatment

with exogenous ceramide or bSMase. The exact mechanism by which ASMase regulates TACE activity is not yet determined, however it seems likely that the effect is on the localization of TACE rather than direct inhibition by ceramide.

An additional role of ASMase in the post-translational regulation of TNFα was also suggested by studies using indirect immunofluorescent: compared to the control cells, in ASMase deficient macrophages more of the internalized proTNFα co-localizes with early endosomes. This genotype-based difference in co-localization of TNFα and EEA1 is exacerbated by the inhibition of TACE mediated proTNFα cleavage, but disappears after the addition of ammonium chloride to block the lysosomal proteolisis of proTNFα. Therefore, one plausible interpretation of these results is that in $asm^{-/-}$ cells the lysosomal proteolysis of TNFα occurs at a slower rate compared to the $asm^{+/+}$ cells, and instead proTNFα is redirected to the recycling pathway and delivered to the plasma membrane where it can be re-used as a substrate for TACE and released as sTNFα in the medium. Such a scenario is in agreement with the detected higher TACE activity in $asm^{-/-}$ cells, and explainsthe increased secretion of sTNFα despite the lack of genotype-based differences at the level of TNFα mRNA.

It is unclear which of the two forms of ASMase is implicated in this phenomenon. While the secretory form might be responsible for the regulation of TACE activity at the plasma membrane, the lysosomal form might be linked to the defects in endosomal maturation (Fig. 5.1.).

Finally, the parallel use of primary peritoneal macrophages and RAW264.7 cell line throughout this study demonstrated that: (i) both cell models responded to LPS stimulation in a similar fashion with regards to the activation of MAP kinases and synthesis of TNFα mRNA, however (ii) they differ substantially in the post-translational processing of TNFα, which might be a result of a different rate of lysosomal proteolysis of uncleaved TNFα. Therefore, RAW264.7 cells may not be a physiologically relevant model to study the mechanisms of TNFα post-translational regulation.

B. Future directions

To decipher the mechanisms by which ASMase influences TACE activity

It has been demonstrated that ASMase and ceramide affect TACE activity, thus it is of great interest to provide additional details regarding the mechanism by which they exert their effects. The existing data suggest that the increased activity in ASMase deficient cells is not a consequence of higher level of TACE expression and most likely ceramide does not directly inhibit the enzyme. However, the difference in the localization of TACE between genotypes and the requirement of an intact membrane for optimal activity of TACE suggest that lipid composition and distribution at the plasma membrane in $asm^{-/-}$ macrophages may create more favorable environment for TACE activity. This hypothesis can be tested initially by *in situ* TACE activity assay where the intact cell membranes will be preserved and will reflect the differences in TACE activity found between $asm^{-/-}$ and $asm^{+/+}$ cells. Furthermore, *in situ* assay performed after cell treatment with sphingomyelinase or cholesterol depleting agents will provide additional insight about the role of sphingomylin:cholsterol enriched lipid domains for TACE activity. A direct link between the increase in TACE activity and its localization within lipid rafts can be established by the isolation of sphingomyelin:cholesterol rich microdomains and measurement of TACE activity in rafts and non-rafts fractions. The distribution of TACE within rafts and non-rafts fractions can be also visualized in macrophages from $asm^{+/+}$ and $asm^{-/-}$ mice by Western blotting .

Translocation of TACE within or out of lipid rafts has been suggested to play a role in substrate selectivity. TNFα is not the only substrate for TACE, therefore it is of importance to determine whether the role of ASMase is limited to TACE-mediated cleavage of TNFα, or it if is a broader phenomenon and holds true for other substrates as well. A number of studies implicate TACE in the shedding of TGFα, therefore measuring the levels of TGFα in medium from $asm^{-/-}$ and $asm^{+/+}$ will provide some insight about the effect of ASMase on substrate selectivity of TACE.

Investigate in further details the role of ASMase in determining the fate of internalized proTNFα

Another question to be addressed is the effect of ASMase deficiency on the distribution of internalized TNFα in cellular compartments involved in the pathways for recycling and degradation. Evidence was provided that after inhibition of TACE-mediated cleavage in $asm^{-/-}$ macrophages more TNFα co-localizes with the marker for early endosome. This result together with the finding that $asm^{-/-}$ have higher amount of early endosomes indicates that in ASMase deficient cells the maturation of early endosomes to late endosomes and lysosomes may be impaired and less TNFα is subjected to lysosomal proteolysis. Experiments for co-localization of TNFα with markers of late endosomes and lysosomes will provide further support for this hypothesis. Additionally, it is also important to provide direct evidence that ASMase deficiency is linked to an increased rate of TNFα recycling to the plasma membrane and that internalized and recycled proTNFα is used for cleavage by TACE. Therefore, it is essential to demonstrate increased co-localization of TNFα with rab11 recycling vesicles in macrophages from $asm^{-/-}$ cells compared to $asm^{+/+}$ cells. To establish a direct link between the rate of the recycling pathway and the amount of sTNFα secreted in the medium, studies with an overexpression of wild-type rab11 or dominant negative form can be performed followed by Western blotting or ELISA to monitor sTNFα in the medium.

Physiological significance of the negative regulation of TNFα production by ASMase and ceramide

The diverse nature of pathogens and the enormous number of proteins engaged in the host defense will lead to overwhelming immune response if it were left unchecked. Therefore, TLR4 signaling is under tight negative regulation, which is achieved at multiple levels and some key players have been already identified. During acute bacterial infection the effective concentration of

LPS is lowered after binding to the soluble TLR4, which appears to serve as decoy receptor and prevents the interaction between TLR4 and other co-receptor complexes, especially CD14 and MD-2, and will therefore terminate TLR4 signaling. However, once the interaction between cell surface TLR4 and LPS has occurred, TLR4 signaling can be controlled by intracellular regulators. The recruitment of a shorter version of MyD88 (MyD88s) ends the signal at the level of TLR4 associated adaptor proteins [137]. Overexpression of MyD88s favors formation of MyD88s-MyD88 heterodimers, in the presence of which IRAK-1 is recruited but not phosphorylated [138]. Interestingly, MyD88s is inhibitory in respect to NF-κB signaling, but its overexpression does not prevent AP-1 activation, suggesting that it is important for the fine-tuning of TLR4 responses [139]. Another candidate for negative regulator of TLR4 signaling is IRAKM, which is induced by LPS and *knockout* mice for this molecule have enhanced production of pro-inflammatory cytokines after stimulation with LPS. Similarly, SOCS-1 deficient mice produce high levels of pro-inflammatory cytokines and are highly susceptible to septic shock, suggesting that SOCS-1 is an important player in negative feedback mechanism for regulation of TLR4-induced responses. Interestingly, however, in none of the above mentioned examples has the exact mechanisms by which these negative regulation occurs been fully elucidated. It appears that some of the intracellular regulators are present constitutively to control TLR4 activation at a physiological level, whereas others are upregulated by TLR4 signaling during infection to attenuate the TLR4 response in a negative feedback loop. ASMase appears to fulfill the requirements of the second type of negative regulators; (i) LPS stimulation leads to activation of ASMase and generation of ceramide, (ii) a hallmark of the immune response to LPS is TNFα production, which in turn exerts strong pro-apoptotic effects on various cell types, and (iii) TNFα induced apoptosis is mediated by ASMase-derived ceramide. Uncontrolled production of TNFα clearly has deleterious effects for the organism and is linked to high morbidity and mortality during septic shock. Therefore, it is tempting to speculate that ASMase might serve as a

sensor and it is a component of a negative feedback mechanism for regulation of TNFα levels.

Both forms of ASMase, secretory and lysosomal, are altered during the onset of various diseases including cancer, obesity, and septic shock. One could speculate that cellular ASMase activity and/or ceramide content might have a more important role in regulating systemic inflammatory responses than previously thought by affecting the magnitude of TNFα release by the cells of the immune system and other TNFα-producing cells. Such a conclusion is supported by studies demonstrating that patients with Niemann-Pick Disease are at high risk of developing atherosclerosis, an inflammatory disease with a clear link to TNFα levels.

A variety of TACE substrates have been described with a broad spectrum of biological activities; so the role of ASMase on TACE activity may not be limited to TNFα secretion and may have additional importance since the differential regulation of TACE expression and activity has been demonstrated in conditions such as cancer and cardiovascular disease. For example, an increase in TACE expression has been found in breast cancer, ovarian carcinomas, and prostatic carcinomas; and it was suggested to play an important role in tumor progression via activation of HER4 ligands [140]. Furthermore, it has been shown that activation of EGFR signaling during breast cancer progression was driven by TACE [141]. Targeting TACE activity has been considered as a promising therapeutic target in colorectal cancer [142]. Furthermore, abnormal TACE activity has also been reported in cardiovascular diseases. For example, a possible role of TACE on vascular complications of diabetes has been suggested [143] and polymorphism of the TACE gene is linked to an increased risk of cardiovascular death [144].

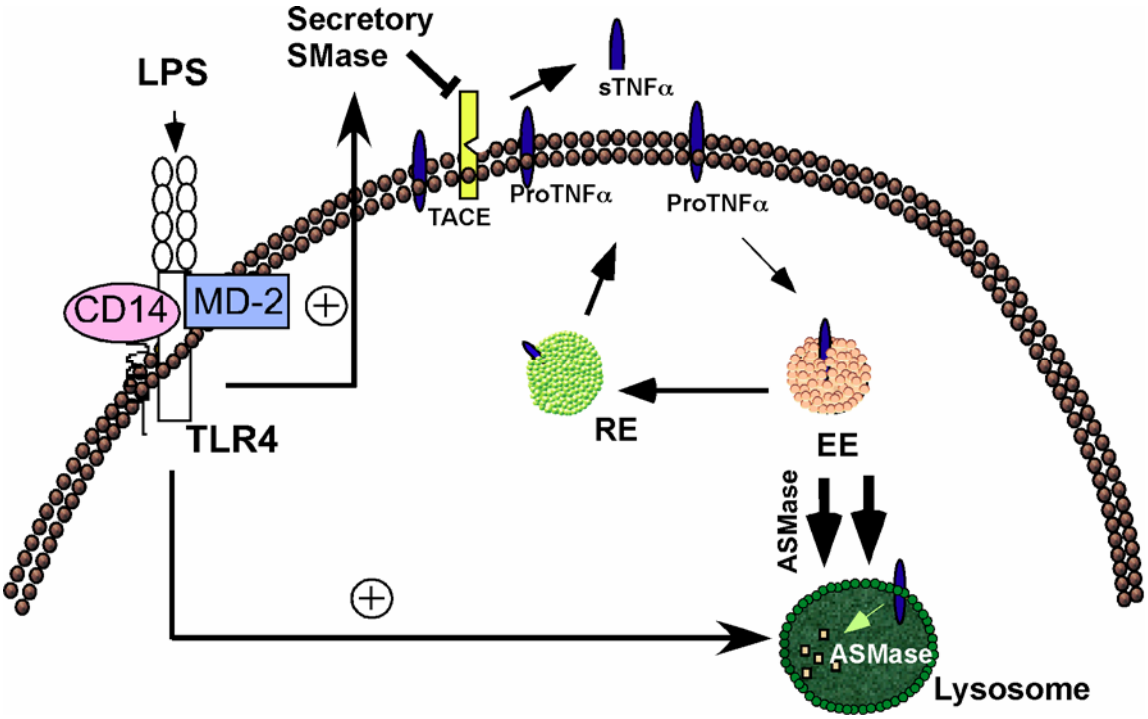


Figure 5.1. Proposed mechanism for the role of ASMase in LPS-induced TNFα production.

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Peer-Reviewed Publications

Monova D, Monov S, **Rosenova K**, Argirova T (2002) Autoantibodies against C1q: view on association between systemic lupus erythematosus disease manifestation and C1q autoantibodies. Ann Rheum. Dis Jun: 61(6): 563-4

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Published Abstracts

Poster presentations

Rozenova, K., (*) Tsacheva, I., Argirova, T., and Kojouharova, M. "Analysis of C1q deficiency and anti-C1q auto-antibodies", 9th Scientific Meeting at Sofia University, November 29-30, 2001

Giltiay, N. (*), Rutkute, K., Karakashian, A**. Rozenova, K**., and Nikolova-Karakashian, M. "Role of Neutral Sphingomyelinase-2 in aging: a molecular switch that regulates cellular responsiveness to inflammation" Symposium on the Biology of Aging: a Multi-Dimentsional Approach Toward Understanding Aging, University of Kentucky, October 9, 2003, Lexington, KY.

Giltiay, N.V. (*), Karakashian, A., Rutkute, K., **Rozenova**, K., and Nikolova-Karakashian, M. "NSMase-2 as a modulator of IL-1β-induced JNK activation during aging" Gill Heart Cardiovascular Research day October 24, 2003, Lexington, KY.

Rozenova, K., (*), Giltiay, N., Boyanovsky, B., and Nikolova-Karakashian, M. "High fat (Western) diet stimulates de novo sphingomyein synthesis in liver and leads to production of LDL with elevated sphingomyelin content" Gill Heart Cardiovascular Research day October 24, 2003, Lexington, KY.

Rozenova, K., (*), Giltiay, N. Boyanovsky, B., and Nikolova-Karakashian, M. "High fat (Western) diet stimulates de novo sphingomyein synthesis in liver and leads to production of LDL with elevated sphingomyelin content" 38th Annual Southeastern Regional Lipid Conference, November 12-14 2003, High Hampton Inn, Cashiers, NC.

Deevska, G., (*), **Rozenova, K.**, Karakashian, A., Rutkute, K., Boyanovsky, B., and Nikolova-Karakashian, M. "Role of S-SMase-generated ceramide in endothelial cell apoptosis during sepsis" 39ttAnnual Southeastern Regional Lipid Conference, November 3-5 2004, High Hampton Inn, Cashiers, NC

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Sathishkumar, S. (*), Boyanovsky, B., **Rozenova, K.**, Giltiay, N., Kudrimoti, M., Mohiuddin, M., Ahmed, M.M., and Nikolova-Karakashian, M. "High dose spatially fractionated (GRID) radiation elevates sphingomyelinase (SMase) activity and ceramide levels in serum: implication for radiation-induced endothelial apoptosis" 51st Annual Meeting of the Radiation Research Society, St.Louis, Missouri, April, 2004 (oral presentation)

Deevska, G., (*), **Rozenova, K.**, Giltiay, N., Boyanovsky, B., Daugherty, A., and Nikolova-Karakashian, M. "Role of Sphingomyelin in fat storage and obesity in mice on a high fat (Western) diet" Gill Heart Cardiovascular Research day October 7, 2005, Lexington, KY.

Rozenova, K. (*), Gergana Deevska Giltiay, N., Boyanovsky, B., Daugherty, A., and Nikolova-Karakashian, M. "Role of Sphingomyelin in fat storage and obesity in mice on a high fat (Western) diet", 40th Annual Southeastern Regional Lipid Conference, November 2-4, 2005, Cashiers, NC

T.V. Getchell, K. Kwong, C.P. Saunders, J. Etscheidt, **K.A. Rozenova**, H. Liu, R.A. Vaishnav, A.J. Stromberg, M.L. Getchell. LEPTIN MODULATES OLFACTORY-MEDIATED BEHAVIOR IN OB/OB MICE Program No.613.13.2005 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2005. Online

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Rozenova, K., (*), Deevska, G., Giltiay, N., Daugherty, A., and Nikolova-Karakashian, M. "Resistance to Dietary Induced Obesity in Acid Sphingomyelinase Deficient Mice" Linda and Jack Gill Heart Institute Cardiovascular Research Day, October 13, 2006 Lexington, KY

Deevska, G., (*), **Rozenova, K.,** Giltiay, N., Wei, J., Smart, E., Merrill, A., Jr., and Nikolova-Karakashian, M. "The rate of sphingolipid biosynthesis in liver affects utilization of palmitic acid for TAG synthesis" Linda and Jack Gill Heart Institute Cardiovascular Research Day, October 13, 2006, Lexington, KY

Rozenova, K. (*), Deevska, G., and Nikolova-Karakashian, M. "The Role of Acid Sphingomyelinase in the regulation of TNF-alpha levels" Linda and Jack Gill Heart Institute Cardiovascular Research Day, October 19, 2007, Lexington, KY

Rozenova, K., (*), Rutkute, K., Deevska, G., and Nikolova-Karakashian, M. "Studies on the regulation of LPS-induced TNFα levels during aging", 42nd Southeastern Regional Lipid Conference, Nov. 7-9, 2007, Cashiers, NC

Rozenova, K., (*), Deevska, G., and Nikolova-Karakashian, M. "ASMase-derived ceramide down-regulates Tumor Necrosis Factor α - converint enzyme (TACE) and the levels of the secretory (p17) form of TNFα. Gordon Research Conference on Glycolipid and Sphingolipid Biology, Feb 17-22, 2008, il Ciocco, Italy

Rozenova, K., (*) and Nikolova-Karakashian, M. "Studies on the role of acid sphingomyelinase and ceramide in TNFα production by macrophages. Physiology Research retreat, August 14-15, 2008, Jabez, KY

Rozenova, K. (*), Deevska, G., and Nikolova-Karakashian, M. "Studies on the role of acid sphingomyelinase and ceramide in TNFα production by macrophages" Linda and Jack Gill Heart Institute Cardiovascular Research Day, October 24, 2008, Lexington, KY

Deevska, G., (*), **Rozenova, K**., Karakashian, A., Rutkute, K., and Nikolova-Karakashian, M. "Biochemical and Functional Studies on SSMase Activity in Mice" 5th International Charleston Ceramide Conference, March 11-14, 2009, Charleston SC.

Invited oral presentations

Rozenova, K. (*), Deevska, G., Giltiay, N, Daugherty, A., and Mariana Nikolova-Karakashian, "Resistance to Dietary-Induced Obesity in Acid Sphingomyelinase Deficient Mice", 41st Southeastern Regional Lipid Conference, Nov. 1-3, 2006 Cashiers, NC. Travel award

Deevska, G., **Rozenova, K.,** Giltiay, N., Wei, J., Smart, E., Merill, A., Jr., and Mariana Nikolova-Karakashian, "The Rate of Sphingolipid Biosynthesis in Liver Affects Utilization of Palmitic Acid for TAG Synthesis, 41st Southeastern Regional Lipid Conference, Nov. 1-3, 2006 Cashiers, NC. Travel award

Rozenova, K. (*), Deevska, G., and Mariana Nikolova-Karakashian, "Studies on the role of acid sphingomyelinase and ceramide in TNFα production by macrophages", 43rd Southeastern Regional Lipid Conference, Nov. 5-7, 2008 Cashiers, NC. Travel award